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Titanium Dioxide Nanoparticles in sunscreens and skin photo-damage. Development, synthesis and characterization of a novel biocompatible alternative based on their *in vitro* and *in vivo* study

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ABSTRACT

Titanium dioxide nanoparticles are widely used in cosmetics, especially in sunscreens due to their capacity to absorb UV harmful wavelengths. However, their biocompatibility remains controversial. In this work, the effect of titanium dioxide nanoparticles, particularly Degussa P25 ($P25TiO_2NPs$) under solar-simulated radiation was studied *in vitro* and *in vivo*. Cell viability and tissue integrity were affected after exposure to $P25TiO_2NPs$ and light for 6 h, showing signs of significant oxidative stress markers and reduced tissue integrity observed by TEM. In order to avoid these undesired effects, a novel biocompatible alternative was presented based on titanium dioxide nanoparticle functionalization with vitamin B2 through a rapid sol-gel method. None of the phototoxicity effects were observed with these functionalized nanoparticles.

Introduction

Nowadays, the use of nanoparticles in pharmaceutical and cosmetic products has increased. In the last case, nano-sized components are used without proper characterization of their effects, leading to unwanted and dangerous consequences for the users [1,2].

The aim of this work was to examine particularly the Degussa P25 titanium dioxide nanoparticles ($P25TiO_2NPs$) because they are among the most employed ones in cosmetics. In fact, all kinds of titanium dioxide nanoparticles (TiO_2NPs) have gained widespread commercialization over recent decades. This white pigment (TiO_2NPs) is used in a broad range of applications, including food, personal care products (toothpaste, lotions, sunscreens, face creams), drugs, plastics, ceramics, and paints. The original source is abundant in Earth as a chemically inert amphoteric oxide, which is thermally stable, corrosion-resistant, and water-insoluble. This oxide is found in three different forms: rutile (the most stable and substantial form), brookite (rhombohedral), and anatase (tetragonal as rutile), of these, both rutile and anatase are of significant commercial importance in a wide range of applications [3]. Additionally, the nano-sized oxide exhibits interesting physical properties, one of them is the ability to act as semiconducting material under UV exposure. In fact, TiO_2NPs are the most well-known and useful photocatalytic material, because of their relatively low price and photo-stability [4]. Although, this photoactivity could also cause undesired molecular damage in biological tissues and needs to be urgently assessed, due to their worldwide use. However, not all nanosized titanium dioxide have the same behavior. In 2007, Rampaul A and Parkin I questioned: "whether the anatase/rutile crystal form of titanium dioxide with an organosilane or dimethicone coat, a common titania type identified in sunscreens, is appropriate to use in sunscreen lotions" [5]. They also

Abbreviations: AOPP, Advanced Oxidation Protein Products; EDS, Energy-Dispersive X-Ray Spectroscopy; MDA, Malondialdehyde; ROS, reactive oxidative species; SEM, surface electron microscopy; TiO_2@NPs, titanium dioxide nanoparticles; TEM, transmission electron microscopy; $P25TiO_2$@NPs, uncoated, Degussa P25 titanium dioxide nanoparticles; VitaminB2@TiO_2NPs, vitamin B2 coated Degussa P25 titanium dioxide nanoparticles.

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suggested that with further study, other types of functionalized titanium dioxide could potentially be safer alternatives. Later, Damiani found that the anatase form of TiO_2NPs was the more photoactive one, and stated that it should be avoided for sunscreen formulations, in agreement with Barker and Branch (2008) [6,7].

In 2017, the Scientific Committee on Consumer Safety (SCCS) warned that they should revise their recommendations if any new evidence emerges in the future related to the potentially harmful effects of TiO_2NPs used in a sunscreen formulation or if they can penetrate the skin. In fact, our work could contribute to this matter because it evaluated the skin penetration of a particular kind of TiO_2NPs. [8]

On the other hand, the U.S. Food and Drug Administration (FDA) in their Final Administrative Order on Sunscreen Drug Products posted in September 2021 still accepts titanium dioxide up to 25% in the list of Generally Recognized As Safe and Effective (GRASE) in the main document, without further clarification on what kind or size of particles [9]. However, on page 24 (Sunscreen containing nanomaterials) FDA clearly "distinguish nanomaterials from other forms of these ingredients'' (zinc oxide and titanium dioxide) and ask for comments on "any particular nanomaterials that you believe should not be permitted for use in OTC sunscreen products". To the best of our knowledge, this Agency did not ban the use of nanoparticulate titanium dioxide in any form, even though it is mentioned on page 34 that the anatase form is the more photoactive one, due to the lack of evidence with real sunscreens OTC (over the counter) *in vivo*. Moreover, other regulations in Latin America (MERCOSUR agreement, 2006) do not state clearly their position on the use of nanoparticulate TiO_2NPs [10].

The lack of clear regulations and controls explains that P25TiO_2NPs are still found in many of the commercialized sunscreens in the market. Some of them are coated to reduce the photoactivity of the anatase form, which is known to be responsible for tissue damage, but not enough studies were made on these coated forms. The anatase photoactivity could trigger the production of reactive oxygen species (ROS) generation, as it was stated before. The ROS are chemically reactive species containing oxygen, such as peroxides, superoxide, hydroxyl radical, and singlet oxygen. They are regularly produced in the biological milieu and counterbalanced by physiological antioxidant defense mechanisms. However, an abrupt increase of ROS may result in non-reversible damage to the skin cells. The effects of coated and uncoated P25TiO_2NPs need therefore to be studied, and articles on this topic present different conclusions. [11–13] Recent literature on this topic found that TiO_2NPs inhalation provokes serious genotoxicity and DNA damage [14–17]. On the other hand, some studies in rats have reported no significant harm to genetic material [18–22].

In order to contribute with experimental evidence that could help to achieve a better understanding of the field for future regulation, in the present work, the biocompatibility of commercial P25TiO_2NPs (one type of TiO_2NPs used in sunscreen formulations) and two novel functionalized P25TiO_2NPs were evaluated under solar simulated irradiation. White light, generated by red, blue, and yellow LEDs, together with UV ones, was chosen to simulate the solar spectra. Functionalization of TiO_2NPs was made with antioxidant vitamins in order to prevent the expected photo-initiated ROS production when nanoparticles are exposed to the simulated solar spectra. Vitamin B2 (riboflavin) and vitamin C were chosen to carry out the functionalization because they are water-soluble, low-cost, and are a constitutive part of biological processes. In addition, it is known that both have the potential to prevent macromolecular oxidation by ROS [23–26].

Experimental

Materials

Nano-sized P25TiO_2NPs were kindly donated by Dr. Scaiano, Ottawa University (Canada). Riboflavin (vitamin B2) was from Sigma and ascorbic acid (vitamin C) and KBr (for IR pills) were from Cicarelli. Base cream for the animal experiments was purchased from Todo Droga and the LED panel was built *ad hoc*.

Irradiation panel

The RGB LED panel was built *ad hoc* for this purpose. Measures: 23.5 × 16.5 cm. Light Intensity: 19,500.10 lux. (43.33 W in 0.2 m^2) when set to solar simulation. It does not produce temperature increases in the surroundings.

Synthesis of vitaminB2@P25TiO_2NPs

The vitaminB2@TiO_2NPs were obtained at room temperature, by a method developed after trying several ratios of reactants. Briefly, 0.02 g of P25TiO_2NPs were dispersed in 1 mL of ultra-pure water and stirred in a Vortex. Next, 200 µl of vitamin B2 dissolved in ultra-pure water (5.3×10^{-3} M) were added to 200 µL of P25TiO2NPs and the mixture was ultrasonicated for 1 hour to achieve a deep-yellow homogeneous suspension. The pellet obtained after centrifuging the suspension for 10 min at 4500 rpm was resuspended in ultrapure water, centrifuged again, and then lyophilized.

The vitaminC@P25TiO_2NPs, on the other hand, were obtained through an optimized method based on Mallakpour et al. [27]. Initially, 0.02 g of P25TiO_2NPs were dispersed in 1 mL of ultrapure water and stirred in a Vortex. Next, 100 µL of HCl (0.01 M) were added (pH 2) to 100 uL of P25TiO_2NPs to avoid gel formation. Then, 100 µL of vitamin C dissolved in ultra-pure water (5.0×10^{-3} M) solution were added to the mixture and was ultrasonicated for 30 min. Finally, vitamin C was added in excess to gain a beige-orange color suspension, and the ultrasonication continued for another 30 min. The pellet obtained after centrifuging the suspension for 10 min at 4500 rpm was resuspended in ultrapure water, centrifuged again, and then lyophilized.

Characterization of vitamins@P25TiO_2NPs

Infrared spectra were performed (from 400 to 4000 cm^{-1}) in vitamins@P25TiO_2NPs samples and the vitamins alone as controls, employing a Nicolet AVATAR 360 Fourier transform infrared spectrophotometer.

UV–vis spectra were carried out in the supernatant of both vitamins@P25TiO_2NPs samples after centrifugation to measure the amount of unbound vitamin. Standard curves at 375 nm and 255 nm were done for vitamin B2 and C, respectively, using a Synergy BioTeK multi-mode microplate reader.

Thermogravimetric analysis (TGA) was conducted in a sample of vitaminB2@P25TiO_2NPs using a TA-THA Q5000 equipment. Temperature ramp rate: 10 °C/min, maximum temperature: 1000 °C, under air. Part of the same sample was mounted on conductive copper tape grids and observed through a Carl Zeiss Sigma scanning electron microscope (SEM) with an EDS probe, at the "Laboratorio de Microscopía y Análisis por Rayos X" (LAMARX) of National University of Córdoba (Argentina).

Assessment of biocompatibility in prokaryotic cells

Testing samples were made mixing 100 uL of TiO_2NPs suspensions (0.2 mg/mL and 0.02 mg/mL) and vitamins@P25TiO_2NPs (0.2 mg/mL and 0.02 mg/mL) with 100 µL ATCC 29,213 methicillin-sensitive *Staphylococcus aureus* (MSSA) (10^7 in PBS, pH 7). Controls were made replacing nanoparticles with the same volume of PBS. The concentrations of nanoparticle suspensions were chosen based on the FDA approved maximal and the minimal amount usually found in sunscreens, which are 20% and 2% (this is equivalent to 0.2 mg/mL and 0.02 mg/mL for nanoparticles suspensions). The cream concentration, on the other hand, was an intermediate value of 10%.

All samples ($n = 6$) were irradiated in a 96 well plate using an LED panel on top for 3 and 6 h before analysis. An identical set of samples

were kept in the dark as controls. The temperature was checked and did not go over 37 °C. The intensity of light was also measured and was constant at 19,500.10 lux. (43.33 W in 0.2 m^2), about 5 times less than actual solar light intensity on Earth's surface Therefore, these findings are indicative of even greater danger in real life.

The cytotoxic effect was tested through the colorimetric assay employing 3′-[1-[(*phenylamino*) -*carbonyl*]−3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT) by reading the absorbance at 490 nm after 3 h of incubation post treatment [28]. The absorbance is proportional to the metabolic rate of viable (live) cells.

ROS were detected through the colorimetric assay employing the nitro-blue tetrazolium salt (NBT salt) by reading the absorbance of the reduced blue molecule.

Macromolecular oxidation was detected in proteins by the colorimetric measurement of Advanced Oxidation Protein Products (AOPP) and in lipids by the colorimetric quantification of malondialdehyde (MDA). Standard curves were run with chloramine-T and 1,1,3,3 tetraethoxypropane (TEP) for AOPP and MDA methods, respectively [29–31]. Values were normalized to initial protein content in samples, measured with Bradford reagent [32]. The standard deviation of at least six measures was calculated and p-value < 0.05 were considered significant.

Assessment of biocompatibility in eukaryotic cells

Hemolysis was studied on suspensions of P25TiO_2NPs (0.2 mg/mL and 0.02 mg/mL), vitaminB2@P25TiO_2NPs (0.2 mg/mL and 0.02 mg/mL) and vitamin B2 (0.2 mg/mL and 0.02 mg/mL) were prepared and mixed with 500 µL of anticoagulated blood (donated by Laboratorio de Hemoderivados, UNC) in a rate of 1/10. A solution of NaCl 10% was used as the positive control and PBS as the negative control. Then, the samples were irradiated using the LED described above for 3 and 6 h to simulate the light penetration into the skin. Also, a set of samples was kept in the dark as control. Finally, the samples were centrifuged and the absorbance at 540 nm was measured in the supernatants. The experiment was reproduced twice; the standard deviation was calculated and p-value < 0.05 were considered significant.

The production of ROS was studied on white blood cells as a model to screen the effect on eukaryotic cells after being exposed to samples and solar simulated irradiation (according to the level of penetration under the skin). For that purpose, the leukocytes were separated from anticoagulated fresh blood using the Ficoll-Hypaque reactive in a well-known technique [33]. Then, 50 µL of suspensions of P25TiO_2NPs (0.2 mg/mL and 0.02 mg/mL), vitaminB2@P25TiO_2NPs (0.2 mg/mL and 0.02 mg/mL) and vitamin B2 (0.2 mg/mL and 0.02 mg/mL) were prepared and mixed with 50 µL of white blood cells suspension. A solution of 3% H_2O_2 was used as positive control and PBS as negative control. Then, the samples were irradiated using the LED panel for 3 and 6 h to simulate the light penetration into the skin. Also, a set of samples was kept in the dark as control. Finally, the ROS were detected through the colorimetric assay employing the nitroblue tetrazolium salt (NBT salt) and the absorbance at 650 nm was measured. The experiment was reproduced twice; the standard deviation was calculated and p-value < 0.05 were considered significant.

Assessment of skin penetration and biohazard in vivo

In order to evaluate the penetration of the nanoparticles, eight adult male Wistar rats (3 months old) were used for the *in vivo* experiments. The protocol was approved by the local University Committee for animal testing and is in accordance with the Canadian Council on Animal Care (CICUAL-RD-2021–892-E-UNC-DEC#FCQ).

Since imaging qualitative studies were made in order to assess skin nanoparticles location and skin morphology under different conditions, only two animals per group were used in order to follow the reduction ethical principle. Then, animals were divided in 4 groups: a-uncoated nanoparticles (P25TiO_2NPs; 10% w/w) with irradiation, b-uncoated nanoparticles (P25TiO_2NPs; 10% w/w) without irradiation, c- vitamin B functionalized nanoparticles (vitaminB2@P25TiO_2NPs; 10% w/w) with irradiation and d-vitamin B functionalized nanoparticles (vitaminB2@P25TiO_2NPs; 10% w/w) without irradiation. Animals were anesthetized with an intraperitoneal administration of the ketamine/xylazine mixture (55/11 mg/Kg) to shave two areas of 5 cm^2 of their backs, in order to expose skin to the cream formulation topic application. In each animal, one area received the complete formulation and the other the cream without nanoparticles as control. Then, animals returned to their home cages and the RGB LED panel (irradiated groups) was located above the lid. After 3 and 6 h, animals were immediately sacrificed and skin areas of interest (with formula or controls) were dissected and preserved for TEM studies.

The RGB LED panel was made *ad hoc,* and configured for solar simulation white light (including the absorption spectra of the nanoparticles: 390–410). No heat was detected at the working distance. The retina of the albino male Wistar rats were not affected under these conditions, because the intensity and time of the applied irradiation was lower than the regular fluorescent lamp bulb in the room (216.65 W/m2) [34].

Results and discussion

Synthesis of vitamins@P25TiO_2NPs

Lyophilized vitamins@P25TiO_2NPs were obtained through the described methods with excellent reproducibility and yield: over 99% of initial P25TiO_2NPs were functionalized.

Solids were stable and did not show visible signs or changes in their spectra after being kept at room temperature for over 60 days. The absorbance at the maximum absorbance wavelength remained unmodified.

Characterization of vitamins@P25TiO_2NPs

Infrared analysis showed that the characteristics bands for the bare nanoparticles are still exhibited in the vitamins@P25TiO_2NPs spectra, such as a wide peak in 450–1028 cm^{-1} related to the stretching vibration of Ti-O-Ti and other peaks in 1630 cm^{-1} and 3400 cm^{-1}, which represent the surface OH groups stretching. The IR spectrum of vitaminB2@P25TiO_2NPs showed signs of binding between compounds. The OH bending peak (1634 cm^{-1}) corresponding to bare nanoparticles disappeared, and the NH_2 bending band characteristic of vitamin B2 appeared (1650 cm^{-1}). The IR spectrum of vitaminC@P25TiO_2NPs also showed signs of successful functionalization. Bands at 1075 cm^{-1}; 1120 cm^{-1}; 1141 cm^{-1} were observed, which are originated by C—O-C vibrations present in the vitamin C. The intense band at 1672 cm^{-1} is attributed to the C = O stretching in the lactone ring while the peak at 1026 cm^{-1} is ascribed to the stretching vibration Ti-O-C. Wide bands at 3880–3600 cm^{-1} are related to stretching vibration OH groups, but those disappear in the modified nanoparticles spectrum. These observations confirm the interactions between the P25TiO_2NPs and the vitamins [35].

The analysis of the supernatant by UV–Vis spectrometry showed that each gram of P25TiO_2NP is loaded with 0.17 g of vitamin B2, after washing them. This value is coherent with the thermogram (Fig. 1), which showed a loss of 19% of weight, attributed to the thermal decomposition of vitamin B2.

The morphology of vitaminB2@P25TiO_2NPs is coherent with the description of Degussa P25 typical population. Size distribution histograms were made from manual measures of the nanoparticles observed in SEM micrographs using ImageJ®. This data showed that more than 70% is anatase (between 20 and 60 nm) with a minor amount of rutile characteristic bars (between 80 and 100 nm) and a small amount of amorphous phase (<40 nm) [36]. Further analysis of the same sample

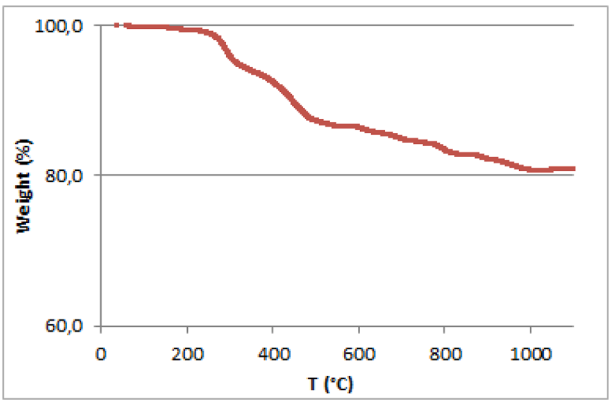

Fig. 1. Weight loss (%) of vitaminB2@P25TiO$_2$NPs.

areas with an EDS probe demonstrated the presence of organic material composed of C and O (Fig. 2). This material was found homogeneously distributed on the surface of the different shapes of P25TiO$_2$NP, not in the background, indicating a specific interaction that could be attributed to the functionalization of the P25TiO$_2$NPs with vitamin B2.

The conjugation of vitamin C to the P25TiO$_2$NPs was confirmed by UV-visible spectroscopy of lyophilized vitaminC@P25TiO$_2$NPs suspensions. The typical absorbance peak of ascorbic acid at 265 nm was found. However, no further characterization was done because they did not show the expected protective effect against the photo-induced cell damage (Fig. 3).

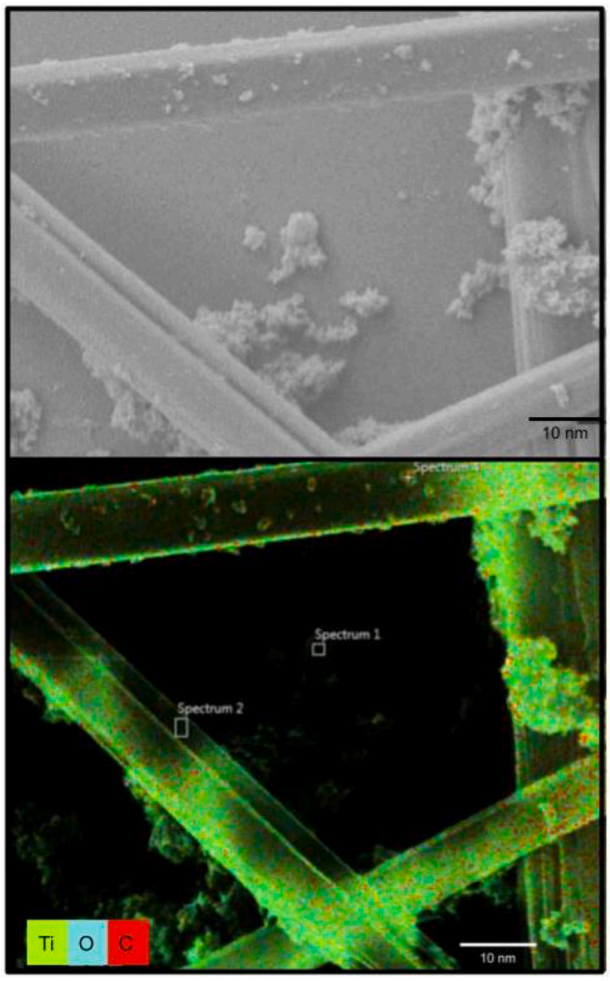

Fig. 2. SEM micrograph and EDS map of the same area of a vitaminB2@P25TiO$_2$NPs.

Assessment of biocompatibility

The toxicity of P25TiO$_2$NPs was evaluated in both prokaryotic (Fig. 3) and eukaryotic cells (Fig. 4). The XTT assay was chosen to measure the cell viability in bacterial cultures of MSSA, a normal skin microbiota microorganism. The reduction in the viability of samples with bare NPs is notorious, possibly due to the described ROS production from the interaction of P25TiO$_2$NPs with light [37]. This effect seems to be avoided when they are functionalized with vitamin B2. Also, the most concentrated vitaminB2@P25TiO$_2$NPs sample (0.2 mg/mL) shows up to 60% more absorbance after 6 h compared to the bare NPs (due to normal cell replication). This may indicate that the antioxidant effect of the vitamin B2 coating is greater than the oxidation damage produced by the NPs. This protective capacity could be attributed to the glutathione redox cycle and the conversion of reduced riboflavin to its oxidized form [38]. Values of cell viability greater than 100% are not rare and could be understood because the XTT assay actually measure metabolic activity when reducing the tetrazole to formazan. It is usually assumed that conversion is dependent on the number of viable cells, but it could also be related to an expected increased enzymatic activity when cells are exposed to small doses of some new substance. Further analysis showed that this effect was not the only one responsible for better cell viability of vitaminB@P25TiO$_2$NPs treated samples.

The vitaminC@P25TiO$_2$NPs, on the other side, did not have any effect on cell protection against ROS. This might be due to the fact that vitamin C, a well-known scavenger of ROS, could behave as prooxidant and even promote ROS and lipid peroxidation [39]. It was recently described that at small concentrations of vitamin C, the prooxidant effects dominate; while in large concentrations the antioxidant ones predominate [40]. The effect also depends on the cell state and the interaction of vitamin C with light. In this case, ascorbic acid may act as an antenna to harvest visible light when conjugated to P25TiO$_2$NPs. Indeed, it was previously found that this combination (in some ratios) could have an improved photocatalytic activity, possibly due to a red shift in its light absorbance [41]. Further studies on vitaminC@P25TiO$_2$NPs were not conducted, because of the poor antioxidant capacity [42].

As mentioned above, these oxide NPs are harmful in part because both anatase and rutile forms are semiconductors and produce ROS. Particularly, P25 kind has band-gap energies estimated of 3.2 and 3.0 eV, equivalent to radiation wavelengths of approximately 388 and 414 nm, respectively. Irradiation at these wavelengths or below produces a separation of charge, resulting in a hole in the valence band and a free

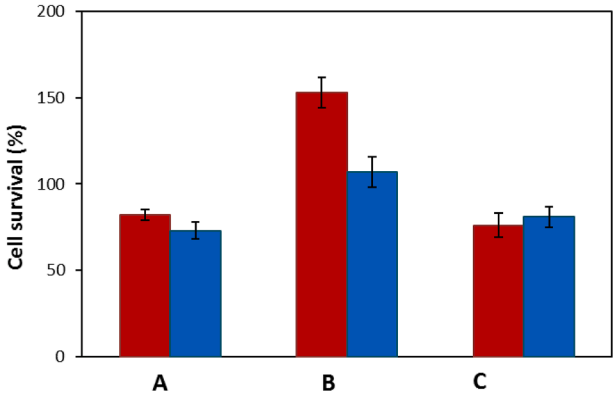

Fig. 3. Cell survival measured on samples of MSSA with bare and functionalized P25TiO$_2$NPs after 6 h of irradiation. A: P25TiO$_2$NPs, B:vitaminB2@P25TiO$_2$NPs, C: vitaminC@P25TiO$_2$NPs in concentrations of 0.2 μg/mL (red) and 0.2 mg/mL (blue). $p < 0.05$.

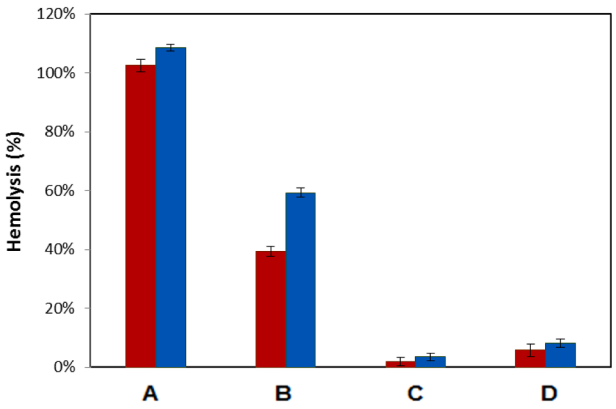

Fig. 4. Hemolysis (%) values of samples, A: 0.2 mg/mL P25TiO$_2$NPs; B: 0.02 mg/mL P25TiO$_2$NPs; C: 0.2 mg/mL VitaminB2@P25TiO$_2$NPs; D: 0.02 mg/mL VitaminB2@P25TiO$_2$NPs after 3 h of irradiation (red) and 6 h (blue). SD <5 for all samples and p <0.05 between C-D and A-B.

electron in the conduction band, due to the electron movement from the valence to conduction bands. These hole–electron pairs generate ROS when they interact with H_2O or O_2 [43,44]. It was described that they can cause an increase in ROS levels after exposure to UV-visible light [45]. The NBT assay in the studied samples showed that bare P25TiO$_2$NPs produce a large amount of ROS, which is drastically reduced by functionalization with vitamin B2 (Fig. 5). This vitamin, also known as riboflavin, was discovered in 1872 as a yellow fluorescent pigment, [46] but its function as an essential vitamin for humans was established more than sixty years later, and its antioxidant capacity was not studied until the end of the XX century [47,48]. This antioxidant role in cells is partially explained because the glutathione reductase enzyme (GR) requires it for good functionality. This enzyme is the one in charge of the conversion of oxidized glutathione to its reduced form which acts as a powerful inner antioxidant and can quench the ROS [49,50]. The cost of this action is that the glutathione is converted to the oxidized form and needs to be recovered by the GR. Consequently, the cells need more vitamin B2. Another glutathione action is the protection against hydroperoxide. This activity is also mediated by riboflavin. Therefore, local delivery of this vitamin seems to significantly help the cells in their fight to keep the oxidative balance, once they are exposed to high levels of ROS.

The ROS seemed to be endlessly produced by P25TiO$_2$NPs upon irradiation, since the values detected after 6 h are similar to the ones after 3 h. However, the amount of vitamin B2 in the surface of the NPs proved to be enough to decrease the ROS detected even after 6 h. Statistical analysis showed a significant difference between C and A. p < 0.05

This constant high rate of ROS production leads rapidly to extreme macromolecular oxidation, here it is observed in the AOPP and MDA detected after 3 h in samples treated with bare P25TiO$_2$NPs (Fig. 6 and Fig. 7). Macromolecular oxidation includes, among others, both protein and lipid oxidation. The ROS causes protein oxidation by direct reaction or indirect reactions with secondary by-products of oxidative stress. Protein fragmentation or cross-linkages could be produced after the oxidation of amino acid side chains and protein backbones. These and later dityrosine-containing protein products formed during excessive production of oxidants are known as advanced oxidation protein products (AOPP). They absorb at 340 nm and are used to estimate the damage to structural cell amino acids. Lipid oxidation is detected by the conjugation of oxidized polyunsaturated lipids with thiobarbituric acid, forming a molecule that absorbs light at 532 nm. Polyunsaturated lipids are oxidized as a result of a free-radical-mediated chain of reactions. The most exposed targets are usually membrane lipids. The macromolecular damage could represent a deadly danger if it is too extensive, and this might be the case. Moreover, it could be observed that cellular damage continues further and becomes irrevocable after 6 h and MDA could not be detected. This may be due to the fact that the lipids were completely degraded and cells were no longer viable. Lipids from the cell membrane are the most prone to oxidation. In fact, lipid peroxidation biomarkers are used to screen the oxidative body balance [51]. At the same time, AOPP values are up to 30 times higher for bare nanoparticles in comparison to the functionalized ones.

This cytotoxic effect was also reported before; *i.e.* Natarajan et al. conducted an experiment that found a strong oxidative stress, morphological changes in mitochondria and substantial loss in the fusion of primary hepatocytes exposed to P25TiO$_2$NPs [52].

The toxicity of P25TiO$_2$NPs under UV radiation could be even higher when combined with other usual components of sunscreens Indeed, Soler de la Vega et al. advise that combination with parabens increases the toxicity of the final cosmetic mixture [53].

Assessment of skin penetration and biohazard in vivo

Both P25TiO$_2$NPs (with or without vitamin B2) were not found beyond the epidermis in 99% of the analyzed TEM images (Fig. 8). This is coherent with previous findings showing that nanoparticles greater than 50 nm can not penetrate the skin, even *in vivo* models with movement, stretching, and friction [54]. However, in one of the zones, a few nanoparticles were observed inside a hair follicle. This could be due to the follicle exposure after the localized rupture of this physical barrier when rats were shaved in order to clean the area for cream topical administration. This finding suggests that nanoparticle-based sunscreen

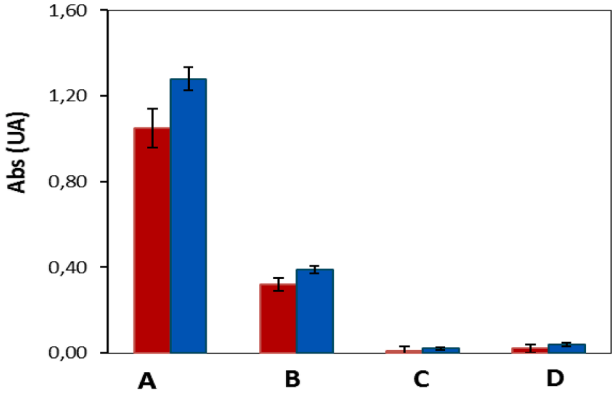

Fig. 5. ROS values (Abs of NBT) in samples of MSSA treated with A: 0.2 mg/mL P25TiO$_2$NPs; B: 0.02 mg/mL P25TiO$_2$NPs; C: 0.2 mg/mL VitaminB2@P25TiO$_2$NPs; D: 0.02 mg/mL VitaminB2@P25TiO$_2$NPs after 3 h of irradiation (red) and 6 h (blue). SD < 0.20 and p < 0.05 between C-D and A-B.

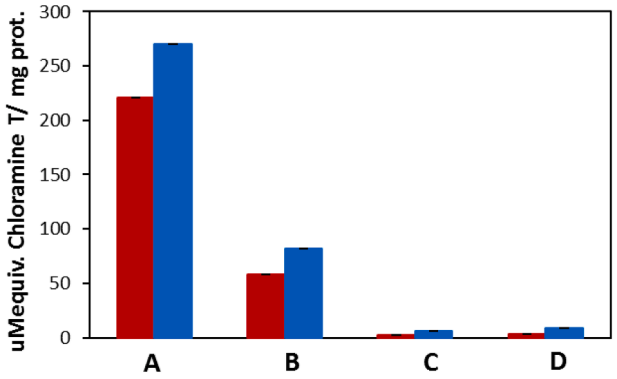

Fig. 6. AOPP measured on samples of MSSA with: A) 0.2 mg/mL P25TiO$_2$NPs; B) 0.02 mg/mL P25TiO$_2$NPs; C) 0.2 mg/mL VitaminB2@P25TiO$_2$NPs; D) 0.02 mg/mL VitaminB2@P25TiO$_2$NPs after 3 h of irradiation (red) and 6 h (blue). SD <1 (error bars too small to be seen) and p < 0.05 between C-D and A-B.

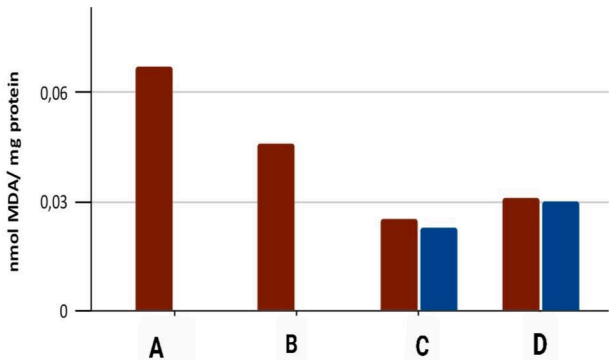

Fig. 7. Lipid peroxidation measured on samples of MSSA with: A) 0.2 mg/mL P25TiO$_2$NPs; B) 0.02 mg/mL P25TiO$_2$NPs; C) 0.2 mg/mL VitaminB2@P25TiO$_2$NPs; D) VitaminB2@P25TiO$_2$NPs 0.02 mg/mL after 3 h of irradiation (red) and 6 h (blue). MDA could not be detected after 6 h of irradiation in a sample with P25TiO$_2$NPs. Error bars are too small to be seen in graphic and $p < 0.05$ between C-D and A-B.

should not be applied on recently shaved or harmed skin, in order to avoid nanoparticle skin penetration.

The integrity of surface skin cells was evaluated with and without solar simulated irradiation. The integrity of the stratum corneum was significantly lower in individuals treated with P25TiO$_2$NPs under the light in comparison to the ones that received the functionalized nanoparticles. Cell membrane suffering is evident (Fig. 9), and it is in accordance with the ROS levels and macromolecule oxidation found *in vitro* for the irradiated P25TiO$_2$NPs. Disruption of the superficial skin layer was observed in all animals treated with no functionalized nanoparticles, under irradiation. This data expands the findings by the group of Professors Fubini and Fenoglio, who showed that P25TiO$_2$NPs "could impact the lipid structure at the top few microns of the stratum corneum" [55]. Control skin under irradiation and without any topic formulation did not show changes in cell structure.

Conclusion

This work confirms previous studies that show P-25 and other untreated anatase 377 nanoparticles should not be employed in sunscreens because the toxicity of P25TiO2NPs under UV radiation is significant.

Here it has been shown that functionalization of P25TiO2NPs with vitamin B2 was able to significantly decrease the oxidative stress produced when they are exposed to sunlight. This finding is of main importance to prevent skin damage and toxicity of sunscreens containing this form of untreated titanium dioxide and should be taken into consideration when updating the regulations mentioned above [56–58].

The rapid method of synthesis described here is readily scalable to the proportions required in cosmetics manufacture.

Data availability statement

Authors would like to mention that aditional experimental details, spectra and pictures are available from the corresponding author on reasonable request.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

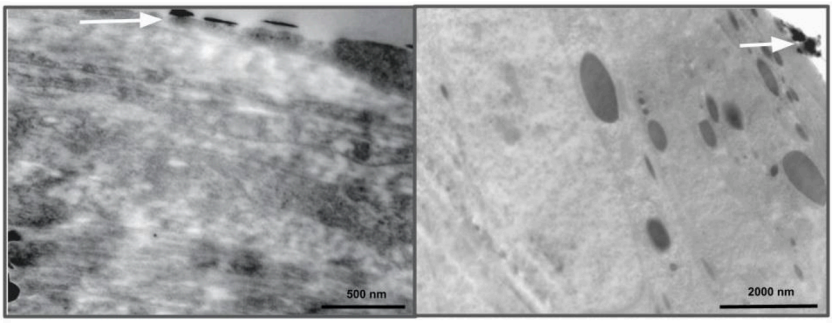

Fig. 8. Selected images of skin stratus treated with P25TiO$_2$NPs 10% (left) and VitaminB2@P25TiO$_2$NPs 10% (right) under light, showing no penetration of the nanoparticles (white arrows) beyond the outer stratum corneum.

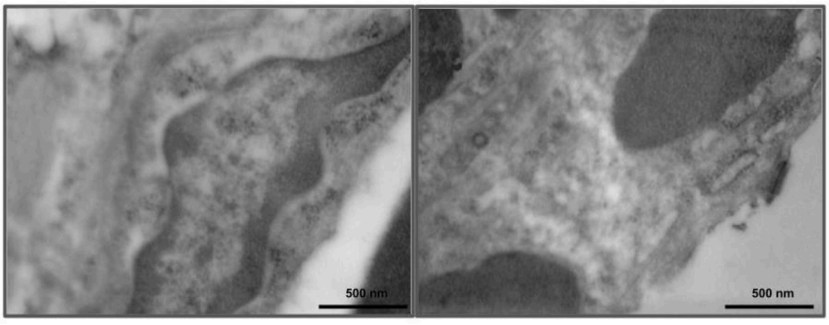

Fig. 9. Selected images of damaged skin treated with P25TiO$_2$NPs 10% (left) and healthy skin treated with VitaminB2@P25TiO$_2$NPs 10% (right).

Data availability

Data will be made available on request.

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