Crystalline Phase Modulates the Potency of Nanometric TiO2 to Adhere to and Perturb the Stratum Corneum of Porcine Skin under Indoor Light

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Crystalline phase modulates the potency of nanometric TiO\textsubscript{2} to adhere and perturb the stratum corneum of porcine skin under indoor light.

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Indoor light
UVA < 1 mW/m²

Nano-TiO₂ → ROS

skin
ABSTRACT

Nanometric TiO₂ is largely employed in cosmetics, but in vitro toxic effects have been reported when nano-TiO₂ is exposed to UV light. The photoreactivity of TiO₂ largely depends on its crystal phase, namely anatase and rutile. Surface acidity, also dependent on crystal structure, may impart a positive or negative charge to nanomaterial surface and ultimately modulate the particle adhesion to tissues. Three nanometric TiO₂ powders with different crystal lattice and surface charge (anatase, rutile and anatase/rutile) have here been employed to investigate the interaction with skin and examine the molecular mechanisms of the TiO₂-induced oxidative damage. The strength of the interaction of nano-TiO₂ with skin has been revealed by chemiometric mapping (μ-XRF and SEM-EDS) after tissue washing. Positively charged anatase and anatase/rutile, but not negatively charged rutile, were strongly held on the skin surface and were able to promote a structural rearrangement of the lipid bilayer in the stratum corneum (DSC and Raman) under controlled indoor illumination (UVA < 1 mW/m²). Under the same conditions, cell-free reactivity tests (ROS-mediated free-radical release and lipoperoxidation) indicated that anatase and anatase/rutile are more reactive than rutile, suggesting a ROS-mediated oxidative mechanism that may alter the structure of the stratum corneum. Both the higher oxidative potential and the stronger adhesion to skin of anatase and anatase/rutile TiO₂ may explain the stronger disorganization induced by these two samples and suggest the use of rutile to produce safer TiO₂-based cosmetic and pharmaceutical products.

KEYWORDS. Nanometric TiO₂ powder; Rutile; Anatase; Skin Adhesion; Oxidative potential; Sunscreen.
1. Introduction

Titanium dioxide is one of the main inorganic nanomaterial currently produced, with a market volume exceeding 10,000 t per year.\textsuperscript{1} Nano-TiO\textsubscript{2} is commonly used as UV filter in sunscreens since it absorbs and scatters a large range of the solar spectrum, including both UVA and UVB.\textsuperscript{2,3} It is also commonly employed as white pigment in lipsticks and make-up products and finds application in composites, cosmetics, food, and pharmaceutical products. TiO\textsubscript{2} in cosmetics is generally considered a safe material\textsuperscript{4} because it does not penetrate into the viable layer of skin, provided that the stratum corneum (SC), the outermost layer of the epidermis that protects underlying tissue, is healthy and intact.\textsuperscript{5} On the contrary, damaged/unhealthy skin is generally considered more permeable and the debate whether or not nanoparticles may penetrate the skin under stress conditions is still open.\textsuperscript{6-11}

Concerns are raised by the charge separation that takes place when TiO\textsubscript{2} particles are irradiated by UV light and photocatalytic reactions occur.\textsuperscript{12-16} In aqueous medium, TiO\textsubscript{2} is in fact active in the generation of several reactive oxygen species (ROS), including hydroxyl radicals ($*$OH), superoxide radical anions ($O_2$), and singlet oxygen ($^1$O\textsubscript{2}).\textsuperscript{17-19} While this property makes TiO\textsubscript{2} a popular photocatalytic reagent in the oxidative removal of organic pollutants,\textsuperscript{20-22} it likely represents a downside in cosmetic and pharmaceutical product formulation. In fact, it is strongly held that the generation of ROS is involved in many detrimental effects on human health induced by several inorganic toxicants.\textsuperscript{23-25} Specifically, ROS generated from UV-irradiated TiO\textsubscript{2} induce genotoxicity and cytotoxicity in human keratinocytes,\textsuperscript{26-28} toxicity on human skin fibroblast,\textsuperscript{29} and collagen degradation in the porcine skin.\textsuperscript{12} To circumvent this intrinsic reactivity, many TiO\textsubscript{2}-based cosmetic products on the market use surface-coated particles to ensure that biomolecules do not come in direct contact with the redox-reactive surface of TiO\textsubscript{2}. However, the stability of these coatings is not always reliable and the complete quenching of TiO\textsubscript{2} reactivity cannot be ensured for long period of time.\textsuperscript{15,30-32} The interaction with skin, \textit{i.e.} the strength of the particle adhesion to stratum corneum, and the chemical reactivity towards biomolecules into the skin are the two key parameters to consider for designing a safer nano-TiO\textsubscript{2} for human use. Since different TiO\textsubscript{2} crystalline polymorphs, including the most commonly used anatase and rutile, exists in nature and
each crystal phase has surface property and different photocatalytic efficiency, the choice of proper crystal phase should enhance protective photoadsorption and minimize oxidative adverse reactions.

This study has been designed to: i) determine the role of the crystal phase in the interaction of nano-TiO₂ with skin, assessing both the strength of adhesion of TiO₂ on the stratum corneum and the structural changes induced in skin, and ii) examine the molecular mechanisms of the TiO₂-induced oxidative damage in skin. Skin sections from pig ear were contacted under indoor illumination with three highly pure commercial nanometric TiO₂ powders bearing different crystal structure, namely anatase, rutile, and anatase/rutile mixed phase. The bulk crystal structure (XRPD, Raman), the size distribution of primary particles, aggregates and agglomerates, and the particle surface charge (ζ potential) have been measured. The adhesion to and the diffusion through the stratum corneum, has been assessed by means of micro X-ray fluorescent chemiometric imaging (μ-XRF) and electron microscopy (SEM-EDS). Raman spectroscopy and differential scanning calorimetry (DSC) have been used to assess the extent of the modification induced by nano-TiO₂ on the stratum corneum structure. The molecular mechanisms involved in the chemical interaction of TiO₂ with skin have been investigated by quantifying the reactivity of the samples towards two model molecules, namely formate ion and linoleic acid by means of the spin-trapping technique (EPR spectroscopy) and colorimetric thiobarbituric acid assay (TBA) respectively. All experiments have been conducted under controlled indoor illumination with very low UVA irradiance. In the continuous strive for providing indications to design safer nano-TiO₂ carried out so far by this group, this is, to the best of our knowledge, the first paper devoted to investigate at the molecular level the different outcomes of the interaction of nanometric TiO₂ polymorphs with skin.
2. Materials and methods

All reagents were from Sigma Chemicals (Sigma-Aldrich S.r.l. Milan, Italy) and all the solutions employed were prepared with Milli-Q ultrapure water system (Millipore S.p.A., Vimodrone, Milan), unless otherwise specified.

2.1. Nanometric TiO₂ powders

Nanometric TiO₂ anatase (Hombikat UV 100, produced by Sachtleben, Germany), rutile (MT500 B, supplied by LCM Trading Milan, Italy), and anatase/rutile (Aeroxide P25, produced by Evonik, Germany) powders were chosen for this study. Bulk and surface properties, were characterized both in air and in the delivery medium (DMSO aqueous solution, 10% \( V/V \)) used to administer samples to the skin.

2.2. Primary particle size (Transmission Electron Microscopy)

The morphology of the samples was investigated in the nanometric range by means of a JEOL 3010-UHR transmission electron microscope (TEM) equipped with a LaB₆ filament operated at 300 kV, beam current = 114 µA and equipped with a 2k × 2k pixels Gatan US1000 CCD camera. Nano-TiO₂ was dispersed in ultrapure water (MilliQ system, Millipore), sonicated for 20 min on a US water bath and a droplet was deposited and dried on lacy carbon Cu grids.

2.3. Aggregate and agglomerate particle size distribution (DLS and Image Analysis)

Dynamic light scattering (DLS) (Zetasizer Nano–ZS, Malvern Instruments, Worcestershire, U.K.) and automatic Flow Particle Image Analysis (Sysmex FPIA-3000, Malvern Instruments, Worcestershire, U.K.) were used to describe the particle size distribution of the TiO₂ powders in the sub-micrometric and micrometric range, respectively. Average hydrodynamic diameter and standard deviation of particle aggregates were determined by using dynamic light scattering (DLS) suspending the powders (50 µg/ml) in a slightly alkaline aqueous solution, in order to enhance the inter-particle electrostatic repulsion and optimize TiO₂ dispersion. Nano-TiO₂ powders were suspended in ultrapure water and
sonicated for 2 minutes with a probe sonicator (4 W/ml, ca. 20 kHz, Sonoplus, Bandelin, Berlin, Germany). To avoid sample heating and solvent evaporation, the TiO₂ suspensions were cooled in 4 °C ice/water bath during sonication. Agglomerate size distribution was measured in the delivery medium with an automatic Flow Particle Image Analysis (FPIA) performed on powders suspended in the delivery medium used. FPIA enables the characterization of both particle shape and particle size distribution in a range of 0.8–300 μm using automated light microscopy and image analysis software. A dilute (0.5 mg/mL) suspension of each sample in DMSO aqueous solution (10% V/V) was passed through a cell where images of the agglomerates are captured using stroboscopic illumination and CCD camera (20× magnification lens). Before image analysis, the suspension was sonicated 30 sec at ca. 2 W/ml.

2.4. ζ potential

ζ potential was evaluated by means of electrophoretic light scattering (ELS) (Zetasizer Nano–ZS, Malvern Instruments, Worcestershire, U.K.). In this technique the velocity of particle in an oscillating electric field, which is proportional to its ζ potential, is measured by light scattering. The ζ potential was measured suspending the nano-TiO₂ in the delivery medium without altering the pH, in order to gather indications on the real surface charge exhibited by the three powders during the skin-interaction experiments. The ζ potential was measured also at several pH values, adjusting the pH of the dust suspension with 0.1 M HCl or 0.1 M NaOH and a plot of ζ potential values versus pH was obtained. The set of experimental points was fitted with a sigmoidal curve (Boltzmann equation) with OriginPro8.0 software suite (OriginLab Corp., Northampton, MA, USA), a simple approximation for the single-site equation model proposed by Sverjensky and Sahai.³⁹

\[
y = A_2 + \frac{A_1 - A_2}{1 + e^{\frac{x-x_0}{\Delta x}}}
\]

where \(A_1\) and \(A_2\) are the lower and upper horizontal asymptotes respectively, \(x_0\) the point of inflection and \(\Delta x\) the curve rate, i.e. the change in \(x\) corresponding to the most significant change in \(y\) values.
2.5. Controlled indoor illumination conditions

To simulate a best-case scenario, all experiments were carried out under controlled indoor illumination with a very low content of UV light. The light irradiance was measured by a portable photoradiometer (Deltahom, Caselle di Selvazzano, Padova, Italy) equipped with two detectors operating in the Vis−NIR range (400−1050 nm) and in the UVA range (315−400 nm). An irradiance of ca. 750 mW/m² (Vis-NIR range) and < 1 mW/m² (UVA) was measured during all experiments. It is worth noting that experiments of TiO₂-induced photodegradation of skin are commonly carried out with an UVA irradiance >10 W/m² (simulating outdoor summer sunlight), about 10’000 times higher than our experimental settings.

2.6. Delivery of nano-TiO₂ on skin

Full thickness pig ear skin was used for experiments using a vertical cells, as proposed by Franz. The skin was rinsed with normal saline and pre-hydrated by floating it with the stratum corneum upward on 0.002% w/v aqueous sodium azide to simulate the in vivo transepidermal hydration gradient. The skin was then sandwiched between two areas of ground glass with the stratum corneum side up. TiO₂ samples, suspended in the delivery medium (DMSO aqueous solution 10% V/V) at 5 mg/ml, were applied to the donor site of the porcine skin surface, which had an available diffusion area of ca. 1.7 cm² (TiO₂ skin concentration = 1.5 mg/cm², that corresponds to the recommended dose for inorganic filter on skin). The contents of the receptor cell (6 ml of water) were continuously stirred and thermostated at 37°C. After 24 hrs, the surface of the skin was thoroughly washed with ethanol and normal saline solution to remove TiO₂ from the surface. TiO₂-contacted skin was collected and all samples were frozen and cut into 30-μm-thick slices in vertical sections with a sectioning cryostat (CM1900, Leica Microsystem, Wetzlar, Germany) and transferred onto glass microscopy slides for micro-XRF and Raman confocal spectroscopy. The skin sections used in this study were obtained without fixative media.

2.7. X-ray Fluorescence Spectroscopy (micro-XRF)
Nano-TiO₂ interaction with skin was assessed by micro-XRF imaging. The washed thin cross sections of untreated and TiO₂-contacted skin were analyzed using an EDAX Eagle III energy-dispersive μ-XRF spectrometer equipped with a Rh X-ray tube and a polycapillary exciting a circular area of nominally 30 μm diameter. The Kα fluorescent line of Ti was integrated pixel by pixel with a lateral resolution of 64×50 pixel (0.155 pixel/μm). The final image resolution was further improved by acquiring spectra from partially overlapping points. Each map was recorded using identical acquisition conditions (40 eV, 1000 μA, dwell time = 5000 msec) and the Ti Kα intensities plotted using a pseudo-color thermal scale (black = 0, white = 100 cps).

2.8. SEM-EDS

SEM observations and EDS analyses were performed using a Stereoscan 410 Leica electron microscope (Oxford Instruments), equipped with a Link ISIS EDS apparatus. The accelerating voltage was 15 kV and the counting live time was 60 s. The skin contacted with nano-TiO₂ was washed, cryo-sectioned, gold-coated and observed without any further fixation.

2.9. micro-Raman spectroscopy

The conformational changes induced on lipids and proteins of the stratum corneum by TiO₂ have been followed investigating the symmetric and asymmetric vibrational stretching modes of CH, CH₂ and CH₃. The laser beam of a confocal Raman microscope (Horiba Jobin-Yvon HR800 and Olympus BX41 microscope) has been focused on a 1 μm × 1 μm wide portion of the SC. A polarized solid state Nd laser operating at a wavelength of 532.11 nm and power of 80 mW and a CCD air-cooled detector operating at -70 °C were used. The use of visible laser beam (λ = 532 nm) as excitation source minimize the oxidative degradation of the skin samples possibly induced by the UV photoreactivity of TiO₂. Calibration of the instruments was performed by measuring the Stokes and anti-Stokes bands of the Si band at 520.7 cm⁻¹. Samples were placed on a polished stainless steel slide and a 50 × objective delivering a power of c. 15 mW on the sample was used. Spectra were acquired with a spectral resolution of ca. 2 cm⁻¹ and an integration time spanning from 100 to 400 seconds. The absence of
structural modification induced by the heat generated by the laser photon flux was evaluated recording and superimposing a time-resolved series of spectra collected from the same tissue spot. To increase the signal to noise ratio and take into account the intrinsic variability of biological tissue, at least 10 spectra per skin sample, collected from distinct portions of the stratum corneum (Figure 4A), were recorded, baseline subtracted, and averaged. Since skin cross section density may vary from sample to sample, the Raman intensity of each spectrum was corrected by normalizing the intensity of the maximum at 2937 cm\(^{-1}\) to 1 Raman arbitrary unit.

2.10. Differential Scanning Calorimetry (DSC)

DSC experiments were performed to study the interaction between the tested formulations and stratum corneum lipid structure. A Perkin Elmer differential calorimeter (DSC7, Perkin Elmer, Nortwalk, CT, USA) equipped with an instrument controller Tac 7/DX (Perkin Elmer) was used. The pig skin samples were treated to part the stratum corneum from the other skin layers, as reported by Yamane.\(^{41}\) The stratum corneum samples obtained were weighed (about 10 mg), hermetically encapsulated in stainless-steel pans and analyzed. A heating rate of 10° C/min was employed in the 25-110 °C temperature range. Data are reported as mean ± SD of at least three independent measures and relative variation of lipid transition enthalpy, i.e. the enthalpy associated to the loss of lipid ordered structure, measured at ca. 70° C were evaluated as follows:

\[
\% \Delta H = (1 - \frac{\Delta H_{sample}}{\Delta H_{blank}}) \times 100
\]

where \(\Delta H_{sample}\) is the enthalpy measured on the skin contacted with nano-TiO\(_2\) and \(\Delta H_{blank}\) the lipid transition enthalpy measured on an untreated section, obtained from the same donor site.

Aqueous suspension of amorphous nanometric SiO\(_2\) powder (Aerosil50, Evonik, 5 mg/ml) that were previously shown to be inert on skin\(^{42}\) was used as negative control, and a mild oxidizing solution, i.e. hydrogen peroxide (0.01 wt%), was used as positive control.

2.11. TiO\(_2\) surface reactivity
In order to assess the reactivity of the TiO₂ samples, the potency to induce the formation of carbon centered free radicals and peroxidate linoleic acid, chosen as a model of stratum corneum lipid, was assessed. All the experiments were carried out under controlled indoor illumination, with UVA irradiance < 1 mW/m², as described above. Reaction with formate evaluates the reactivity of oxidizing “holes” (h⁺) occurring at TiO₂ surface. The direct or singlet oxygen-mediated interaction with H-C bond in formate ions may generate a carbon-centered free radical. The reactivity of nano-TiO₂ towards linoleic acid quantifies the potency of the different TiO₂ polymorphs to promote the lipid peroxidation chain reaction, a well-accepted marker for particle-induced cell membrane damage.

**Generation of carbon centered radical.** Formate ion is a simple - yet largely adopted - target molecule suitable to assess the reactivity of an inorganic solid in heterogeneous phase. Anatase, rutile and anatase/rutile TiO₂ (30 mg) were suspended in 0.5 ml of sodium formate (1 M) buffered solution (potassium phosphate buffer, 125 mM, pH 7.4) in the presence of DMPO (5,5-dimethyl-1-pyrroline-1-oxide, 75 mM, Alexis Biochemicals, San Diego, CA) as spin-trapping agent. The suspension was continuously stirred and exposed to indoor light during the experiment. The EPR spectra were recorded on 50 μL of the suspension withdrawn after 10, 30, and 60 minutes. The experiments were repeated at least three times. Splitting constants of the signal recorded: \( a^N = 1.56 \text{ mT} \quad a^H = 1.87 \text{ mT} \).

**In vitro lipoperoxidation (TBA assay).** The potential of nano-TiO₂ to induce lipid peroxidation was assessed by means of thiobarbituric acid (TBA) assay using linoleic acid as a model of the polyunsaturated fatty acids in the cell membrane. The main lipoperoxidation product malonyldialdheyde (MDA) forms with TBA a colored complex. The assay is based on the reactivity of MDA - a colorless molecule - with TBA to produce a pink adduct which absorbs at 535 nm. TiO₂ was suspended (15 mg/ml) in a buffered (potassium phosphate buffer, 10 mM, pH 7.4) dispersion of linoleic acid (1 mM) containing 2.5 % of ethanol. The suspension was continuously stirred under controlled indoor illumination at RT for 72 h. The lipid peroxidation was stopped by adding 0.1 ml of an ethanolic solution of butyl hydroxyl toluene (BHT, 0.2%) to the suspension. The powder was removed by centrifugation (20,000 RPM for 30 min). A solution of TBA (0.034 M) containing HCl (0.25 M) and
trichloroacetic acid (TCA, 0.92 M) was added to the supernatant (2:1 vol:vol) and the resulting solution was heated at 100°C for 1 h. After cooling in an ice bath, 3 ml of 1-butanol were added to extract the colored complexes. The absorbance at 535 nm was measured on the organic phase by means of a UV/Vis spectrophotometer (Uvikon, Kontron Instruments, Inc., Everett, MA, USA). The experiments were repeated at least three times.

2.12. **Statistical analysis**

When necessary, data were statistically analyzed by means of ANOVA with post-hoc Tukey’s test. SYSTAT (version 10; SYSTAT, Evanston, IL, USA) was used to perform the tests and p<0.05 was considered significant.
3. Results

3.1. Physico-chemical features of nano-TiO₂

The chemical nature and the occurrence of different crystalline phases of the three TiO₂ samples, were identified by means of XRF, XRPD. The occurrence of each crystal phase, calculated in previous works,\textsuperscript{32,45} was carried out through the Rietveld refinement of the TiO2 diffractograms carried out with MAUD\textsuperscript{46} software suite and further confirmed in this work by Raman spectroscopy (Figure S1 in the Supporting Information). Nanometric TiO2 anatase and rutile are 100% constituted by the single phase of TiO2 polymorph. The composition of the mixed phase is about 1:9 anatase:rutile. No chemical elements other than Ti and O were detected by elemental analysis.

3.2. Particle size distribution and aggregation/agglomeration of nano-TiO₂

Particle size distribution (PSD) of each sample was measured in order to evaluate both the primary particle size, i.e. the dimension of the individual TiO₂ crystallite, and the size of aggregates and agglomerates which may form in aqueous suspension. Figure 1 shows: TEM images (first row), diameter distribution of aggregates performed under alkaline condition to ensure the best particle dispersion (DLS analyses, second row), and agglomerate diameters in delivery medium (automated image analysis, third row) of the three TiO₂ samples. TEM gives evidence of the nanometric nature of the three samples. Anatase TiO₂ exhibits the smallest primary particle size (ca. 4 nm), whilst both rutile and anatase/rutile are bigger (35 and 25 nm, respectively). The DLS intensity plots describe quantitatively the hydrodynamic diameter ($d_{h}$) of particle aggregates – i.e., those particles gathered together through covalent bonds, thus virtually not separable with a mechanical action. Anatase aggregates show a bi-modal distribution, having a smaller average $d_{h}$ of about 100 nm, and a second larger size of ca. 500 nm. By means of an automated image analysis system operated in wet condition the circular equivalent diameter ($d_{E}$) of the agglomerates, i.e. those particles associated by low electrostatic/Van der Waals forces, formed by nano-TiO₂ suspended in the skin delivery medium, was also investigated. In the delivery medium, the size of agglomerated TiO₂ ranges from few to tens of
microns. In particular, anatase forms the smaller agglomerates with a size range of 1-30 µm, while rutile and anatase/rutile have comparable agglomeration patterns with sizes ranging from 4 to 100 µm.
Figure 1. Particle size distribution and aggregation/agglomeration of nano-TiO₂. TEM micrographs (upper row), hydrodynamic diameter of aggregates (DLS plot, middle row) and equivalent diameter of agglomerates (automated image analysis, lower row) of TiO₂ anatase (a), rutile (b) and anatase/rutile (c) in vacuum, in DI water, and in the delivery medium (TEM, DLS and image analysis, respectively). In the DLS plot, the asterisk highlights the size of primary particle as evaluated by TEM. Automated analysis of particle size distribution is reported for TiO₂ anatase (a), rutile (b) and anatase/rutile (c). Particle count (squares) and cumulative size distribution (line) are superimposed on each plot.
Detailed description of the primary particle size (PPS) distribution, the Z-average hydrodynamic diameter ($d_{H}$) in water at pH 9, and the agglomerate circular equivalent diameter in the delivery medium ($d_{E}$) are reported in Table 1. From the automated image analysis, also a cumulative distribution of agglomerate circular equivalent diameter was plotted and the 25th, 50th, and 75th quartile reported. In the delivery medium, anatase exhibits $d_{E}$ average values smaller than rutile and anatase/rutile, namely 7.5, 25.5, and 19.6 μm respectively. In the delivery medium, particle size dispersity has also been analyzed by calculating the difference between the 25th and 75th quartile, normalized by the median distribution value (50th quartile). Dispersity data (Table 1) indicate that anatase and rutile have a similar size dispersity (0.85 and 0.83 respectively), while agglomerate sizes vary in a shorter range for anatase/rutile, dispersity = 0.71.

Table 1. Particle size distribution (PSD) of the nano-TiO$_2$. Primary particle size (PPS) was measured by TEM; aggregate size was investigated by DLS, adopting the best dispersion conditions (DI water, pH > 9); and agglomerate size was measured by automated image analysis system operated in a wet environment, i.e. suspending the powders in the delivery medium. The average PPS (nm), the average hydrodynamic diameter of aggregates and the first (25%), second (50%, median), and third (75%) quartile for agglomerate are reported. The number of particles counted and measured by image analysis software is also reported.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Commercial name, supplier</th>
<th>Average PPS (nm)</th>
<th>Z-average hydrodynamic diameter (nm) in water at pH 9</th>
<th>Agglomerate equivalent diameter in the delivery medium (μm)</th>
<th>25% (Q1)</th>
<th>50% (Q2)</th>
<th>75% (Q3)</th>
<th>Dispersity (Q3-Q1)/Q2</th>
<th>measured particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anatase</td>
<td>Hombikat UV100 Sachtleben, DE</td>
<td>3.9±0.1</td>
<td>291.1±56.6 (PDI = 0.3)</td>
<td></td>
<td>4.8</td>
<td>7.5</td>
<td>11.2</td>
<td>0.85</td>
<td>4,558</td>
</tr>
<tr>
<td>Rutile</td>
<td>MT500 B LCM Trading, IT</td>
<td>31±2</td>
<td>137.7±3.8 (PDI = 0.1)</td>
<td></td>
<td>15.8</td>
<td>25.5</td>
<td>37</td>
<td>0.83</td>
<td>4,189</td>
</tr>
<tr>
<td>Anatase / Rutile</td>
<td>Aerioxide P25 Evonik, DE</td>
<td>30±4 (A) 50±6 (R)</td>
<td>214.5±2.6 (PDI = 0.2)</td>
<td></td>
<td>13.5</td>
<td>19.6</td>
<td>27.5</td>
<td>0.71</td>
<td>16,369</td>
</tr>
</tbody>
</table>
3.3. \( \zeta \) potential and point of zero charge (PZC)

Since the surface charge of a particle is one of the key physico-chemical features relevant for understanding the interaction of a nanomaterial with biological matter\(^47\) – in the present case the skin – the \( \zeta \) potential and the point of zero charge (PZC) for the three TiO\(_2\) have been evaluated (Figure 2). When nano-TiO\(_2\) is dispersed in the delivery medium, the pH of the water suspension becomes slightly acidic, namely 5.0, 5.5, and 5.4 for anatase, rutile and anatase/rutile, respectively. Noticeably, under these experimental conditions, anatase and anatase/rutile \( \zeta \) potentials values are +11.5 V and +9.1 V, while rutile is negatively charged with a \( \zeta \) potential of −16.7 mV. For TiO\(_2\) the PZC value can be associated to the acidity of surface moieties\(^47\) and can be readily calculated from the \( \zeta \)-plot (\( \zeta \) potential vs. pH). Like many transition metal oxide, TiO\(_2\) surface is amphoteric in nature. In a simplified yet practical approach to PZC, the acidity of polarized groups exposed at the surface, namely “titanol” (>TiOH), can be correlated to the point of zero net proton charge. In this single-site model, the pH of the surrounding media directly determines the charge of the surface following the equilibrium:\(^39\)

\[
>\text{TiOH} + >\text{TiO}^- + 2 \text{H}^+ \rightleftharpoons >\text{TiOH}_2^+ + >\text{TiOH}, \quad K_{\text{PZC}}
\]

The PZC is 5.6, 4.3, and 6.2 pH units for anatase, rutile and anatase/rutile respectively, in good agreement with previous experimental\(^48\) and calculated\(^49\) values. The data indicate a more acidic surface of rutile by respect to anatase and anatase/rutile.
Figure 2. $\zeta$-potential of TiO$_2$ anatase, rutile and anatase/rutile suspensions in delivery medium (DMSO aqueous solution 10% $V/V$) as a function of pH. The empty points represent the mean values ± SD of five different measurements. The point of zero-charge (PZC) is highlighted on each plot with a full circle. The $\zeta$-potential of nano-TiO$_2$ suspended in the delivery medium without adjusting the pH is indicated on each plot with a full star.

3.4. Nano-TiO$_2$ interaction with the skin

To investigate the interaction of TiO$_2$ with the skin, the occurrence of Ti on ear skin sections was quantified by means of a $\mu$-XRF spectroscope. Such technique allows direct visualization of unlabeled nano-TiO$_2$ particles in the ppm range, with a spatial resolution of few microns. Figure 3 reports the light microscopy images of the thin skin cross sections (left panel), with the SC evidenced by an overlaid dotted line, and the pseudo-thermal colorimetric mapping of the same area integrated for the intensity of Ti K$\alpha$ fluorescent line (right panel). The X-ray fluorescence of Ti was almost continuously detected on the uppermost portion of the skin samples contacted with anatase and anatase/rutile. This implies that a significant amount of anatase and anatase/rutile TiO$_2$ (Fig. 3A’ and C’) strongly adhere to the SC and
was not removed by the washing procedure. On the contrary, rutile was efficiently removed by the washing performed on the skin after exposure. Since the X-ray fluorescence signal due to Ti was not detected on the rutile-contacted SC, we can confidently assume that its residual concentration in the skin, if any, is lower than 10 ppm. In all cases, regardless to crystalline phase, nano-TiO₂ was not observed in the deep layers of the skin, or even only underneath the stratum corneum. This was further confirmed by SEM imaging and elemental analysis (EDS) and reported in Figure 4. The absence of dermal transmigration of TiO₂ through pig skin is consistent with previous findings on different skin models⁹ and in vivo.⁶ TiO₂ was not even detected in the proximity of follicles, opposite to what suggested on the possible transmigration through the SC via a follicular pathway.⁵⁰ However, this could be due to the ex vivo experimental set up adopted in this work, including the absence of the “pumping effect” that massage-induced hair movements can have on sub-micrometric aggregates when administered in vivo.⁵⁰ It has been reported that only nanoparticles with diameter < 10 nm⁵ may pass the SC barrier and diffuse through the skin. It is worth noting that present findings rule out the penetration of nano-TiO₂ even if primary particle size is smaller than 10 nm –anatase has a PPS of ca. 4 nm– provided that particle aggregation occurs.
Figure 3. Chemiometric mapping of TiO$_2$ NPs in skin. The occurrence of TiO$_2$ in the thin cross sections of pig skin contacted with anatase (A), rutile (B), and anatase/rutile (C) photographed at 100$\times$ magnification were analyzed by means of XRF obtaining a fake-color image of the Ti in the skin, with a lateral resolution of 64$\times$50 pixel (A’, B’, and C’). Normalized ROI intensity of Ti K$\alpha$ fluorescent line was reported adopting a pseudo-thermal scale bar. The stratum corneum is highlighted with a dotted line in the optical image and lines are drawn to help reader’s eyes also in the chemiometric map.
The interaction of nano-TiO$_2$ with skin, observed with micro-XRF on large, millimetric, portion of the sample, was further investigated by means of SEM-EDS, taking advantage of the higher spatial resolution of electron microscopy.

A section of skin contacted with anatase/rutile suspended in the delivery medium is imaged and analyzed in Figure 4. Similar results have been found for anatase (Figure S2). Rutile-contacted skin did not show any detectable occurrence of TiO$_2$. The presence of TiO$_2$ in the SC was evidenced by the Ti EDS peak throughout the whole SC full thickness (Figure 4, spots A). The intensity of the Ti signal is suddenly almost undetectable underneath the SC (Figure 4, spot B) and no TiO$_2$ is detected in the deeper layers of the skin (spot C), confirming the inability of the nano-TiO$_2$ anatase/rutile analyzed to penetrate the integer skin and reach the viable epidermis. Surprisingly, no aggregates/agglomerates were found adhering on the external surface of the stratum corneum (SC). This suggests that the nano-TiO$_2$ may diffuse into the SC, following dissolution of larger agglomerates into the SC structure. It is worth noting that anatase/rutile primary particle size (ca. 25 nm) is not compatible with a simple diffusion mechanism (free energy driven), which occurs for particles/molecules $< 10$ nm$^5$, but requires that large TiO$_2$ particles induce disorganization of the lipid structure of the SC, provoking an increase in SC permeability.
Figure 4. **SEM imaging and EDS analysis of the stratum corneum.** Skin section of anatase/rutile contacted sample was analyzed by means of SEM-EDS. Secondary electron (SE) imaging of the uppermost layer of the skin section is reported. An arrow highlights the full thickness of the SC. Spot EDS analyses were performed on skin section and EDS spectra are reported (left, inset). Overlaid letters clarify the correspondence between each EDS spectrum and relative analysis spot on the skin.

3.5. **Raman investigation of the stretching mode of lipids and proteins in the stratum corneum**

Raman spectroscopy is a valuable tool for investigating in vitro and in vivo cells and tissues.\(^{51}\) It has been successfully used to detect spectral changes in malignant and pre-malignant cells,\(^{52}\) to elucidate the conformational alterations induced by solvent on the secondary structure of keratin in isolated corneocytes,\(^{53}\) and to investigate the stratum corneum arrangement in psoriatic skin.\(^{54,55}\) The following conformational changes induced on lipids and proteins of the SC by TiO\(_2\) have been here followed investigating the symmetric and asymmetric vibrational stretching modes of CH, CH\(_2\) and CH\(_3\) moieties which constitute the aliphatic chain in lipids and proteins.
The laser was focused on distinct portions of the SC (Figure 5A) and Raman spectra from untreated skin and skin contacted with rutile, anatase and anatase/rutile are reported in Figure 5B (spectrum a, b, c, and d, respectively). The Raman band assignment is proposed after Anigbogu and coworkers: 56

\[ \nu(CH) \text{ aliphatic at } 2719 \text{ cm}^{-1}, \nu_s(CH_2) \text{ at } 2852 \text{ cm}^{-1}, \nu_d(CH_2) \text{ at } 2883 \text{ cm}^{-1}, \nu_s(CH_3) \text{ at } 2937 \text{ cm}^{-1}, \nu_d(CH_3) \text{ at } 2974 \text{ cm}^{-1}, \nu(CH) \text{ olefinic at } 3060 \text{ cm}^{-1}. \]

The various modes can also be selectively ascribed to proteins and lipids, being the \( \nu(CH) \) aliphatic, \( \nu_s(CH_2) \), and \( \nu_d(CH_2) \) mostly due to lipids and \( \nu_s(CH_3), \nu_d(CH_3), \) and \( \nu(CH) \) olefinic predominantly associated to proteins. 56 The spectra show a relevant difference in the \( CH_2 \) region ascribed to lipids vibrational features. In particular, the Raman spectrum from the stratum corneum of untreated skin (a) is almost superimposable with the spectrum from rutile-contacted SC (b), while an increase of the relative intensity of the \( CH_2 \) stretching modes (both symmetric and asymmetric) is clearly observed for skin contacted with anatase (c) and anatase/rutile (d). This is consistent with a partial structural rearranging of the lipidic chains. In fact in lipids, the ratio of the symmetric methylene \( C-H \) stretching-mode intensities (peak height \( I_{2890}/I_{2850} \)) is sensitive to the packing order of the acyl chains and the observed increase of the intensity of the 2850 \( \text{cm}^{-1} \) band with respect to 2890 \( \text{cm}^{-1} \) may account for a decrease of the intermolecular order of lipids. 57

This phenomenon, occurring with anatase and anatase/rutile, but not with rutile, may be due to physical interaction of nano-\( \text{TiO}_2 \) with the stratum corneum lipids or to some \( \text{TiO}_2 \)-induced chemical oxidative damage. 58-60
Figure 5. Raman spectra of the CH stretching band of lipid and proteins in the stratum corneum.

Thin cross sections (A) were analyzed focusing the laser beam (a) on an intact portion of the SC (b). The border between SC and viable epidermis (c) is clearly visible. The Raman spectra (B) from untreated (negative control, spectrum a) and rutile–, anatase– and anatase/rutile–contacted skin (spectra b, c, and d) are reported. At least 10 independent spectra were recorded, baseline subtracted, averaged, and normalized setting the maximum intensity to 1 AU.

3.6. Transition enthalpies of lipids in the stratum corneum

The modification induced by nano-TiO₂ suspensions on the lipids were investigated in the stratum corneum by measuring the enthalpy variation in reaching a disordered structure upon heating (DSC).
The enthalpy data are reported in Figure 6. The skin was treated to part the stratum corneum from the other skin layers and the enthalpy associated to the lamellar-disordered structure transition at ca. 70° C was evaluated before and after treatment with TiO₂ aqueous suspensions (5 mg/ml). The relative decrease in enthalpy (ΔH%) of the lipid thermal transition in the presence of TiO₂, compared to transition enthalpy of skin treated with negative control (nano-SiO₂ powder), indicates that nano-TiO₂ perturbs the intercellular lipid packing arrangement of the stratum corneum. The anatase and anatase/rutile perturbed the intercellular arrangement of the lipid bilayer to a higher extent, close to that caused by the positive control (H₂O₂). DSC data also indicate that also rutile induces in the SC a significantly higher perturbation than the negative control, but well below the positive control.

![Graph](image)

**Figure 6. Variation of the lipid transition enthalpies in the stratum corneum of TiO₂ contacted skin.** Anatase (A), rutile (R), and anatase/rutile (A/R) NPs (5 mg/ml) were contacted overnight with pig skin section. The lipid transition enthalpy normalized by the transition enthalpy of untreated skin was compared to a negative and positive control (amorphous nano-SiO₂ powder and hydrogen peroxide, respectively). Data are reported as mean ± SD of at least three independent measures. According to Tukey’s test (ANOVA, p<0.05), bars which do not share at least one letter are statistically different.

### 3.7. Generation of carbon-centered radical and cell-free lipoperoxidation assay
The well-known photogeneration of charge carriers in TiO$_2$, producing both electrons ($e^-$) and holes ($h^+$), promotes, in water, the formation of ROS.$^{19,26}$

(1) $e^- + O_2 \rightarrow O_2^{•-}$

(2) $h^+ + H_2O \rightarrow OH^- + H^+$

(3) $h^+ + O_2^{•-} \rightarrow ^1O_2$

Reaction with dissolved oxygen and water (Eqn. 1 and 2) can be monitored and quantified by measuring the capability of TiO$_2$ to generate a carboxyl radical ($•CO_2^-$) from a stable organic precursor (formate anion, HCO$_2^-$), following both a ROS-mediated or surface-assisted reaction mechanism (Eqn. 4).

(4) $h^+ + HCO_2^- \rightarrow •CO_2^- + H^+$

Such radical can be detected via the spin trapping technique and EPR spectroscopy, provided that concentrated HCO$_2^-$ and neutral buffered solutions are employed. Despite its slower kinetics, due to the low molar concentration of $O_2^{•-}$, the formation of singlet oxygen ($^1O_2$) by the recombination of a superoxide radical anion with an oxidative hole ($h^+$) may also take place on the TiO$_2$ surface (Eqn. 3).$^{18}$ Singlet oxygen is a very reactive species capable to start the lipoperoxidation, a complex process where unsaturated fatty acids and lipids are degraded to a variety of products and a well-accepted marker for particle-induced cell membrane damage.$^{44}$ Lipid peroxidation may also be mediated by hydroxyl radicals through the formation of carbon-centered radical of fatty acids.

Figure 7A shows the EPR spectra recorded after 60 minutes of incubation of formate ion with the same amount of anatase, rutile, and anatase/rutile TiO$_2$ (A, R, and A/R respectively). The typical six lines EPR spectrum of the DMPO-CO$_2^{•−}$ adduct was detected with all the three samples indicating the capability of nanometric TiO$_2$ to cleave C–H bond. It is worth noting that such reactivity was here measured carrying out the reaction under indoor light (UVA < 1 mW/m$^2$).

The signal intensities, which are proportional to the amount of carboxyl radical produced, were similar and rather intense for anatase and anatase/rutile, whilst the signal recorded with rutile suspension exhibits a lower intensity. Further differentiation among the samples is highlighted by the kinetics of the
•CO$_2^-$ radical release (Figure 6B). The signal of the radical adduct was recorded at 10, 30 and 60 minutes and double integrated. The intensity of the signal at 10 min. is negligible for all the samples, whilst after 30 min of incubation only anatase and anatase/rutile exhibit a significant radical activity. After 60 min also rutile induces a detectable generation of •CO$_2^-$ radical, and the following intensities were calculated as follows: anatase 381±57; rutile 237±8; and anatase/rutile 319±23, with rutile having a radical reactivity significantly lower that anatase and anatase/rutile. The same experiment was carried out in the absence of the dust and no EPR signal was detected at each time points.

**Figure 7. Generation of carbon-centered radicals under indoor illumination.** A) EPR spectra recorded on the suspension of anatase (A), rutile (R), and anatase/rutile (A/R) in a solution of 1 M of sodium formate and 0.085 M DMPO in 0.25 M phosphate buffer, pH 7.4, after 1 h of incubation under indoor illumination. B) Kinetics of •CO$_2^-$ release. Intensity of the signal recorded after 10, 30 and 60 minutes of incubation of anatase (A), rutile (R), and anatase/rutile (A/R).

The potential of anatase, rutile and anatase/rutile TiO$_2$ to induce lipid peroxidation was assessed by means of thiobarbituric acid (TBA) assay using linoleic acid as a model molecule. To take into account any antioxidative processes taking place during the peroxidation of linoleic acid in the presence of O$_2$, the same experiment was carried out on a blank emulsion of linoleic acid in the absence of TiO$_2$. In Figure 8, the amount of MDA detected in solution after 72 hours of incubation of linoleic acid with TiO$_2$ is reported. The three nano-TiO$_2$ showed a significant ability to induce lipid peroxidation in cell-
free system, kept at 37°C under indoor illumination. The lipoperoxidation induced by anatase and anatase/rutile is significantly higher (0.298±0.016, and 0.257±0.011 A.U., respectively) than that induced by rutile (0.152±0.024 A.U.), suggesting that anatase and anatase/rutile may have a higher potential to induce peroxidation towards cell membranes than rutile.

![Graph](image)

**Figure 8. In vitro lipoperoxidation under indoor illumination.** Absorbance (λ = 535 nm) recorded on the supernatant after incubation of the anatase (A), rutile (R), and anatase/rutile (A/R) suspended in a 1 mM suspension of linoleic acid in 5 mM phosphate buffer (pH 7.4) for 72 h under indoor illumination. A linoleic acid suspension without dust was employed as blank. The data are expressed as the mean value of three separate determinations ± SD. Vs blank: *p < 0.05; R vs. A and R vs. A/R: # p < 0.05.
4. Discussion

The three nano-TiO$_2$ samples with different crystal structure, namely anatase, rutile, and anatase/rutile, share key physico-chemical properties relevant to establish interaction with tissues: nanometric primary particles, sub-micrometric aggregates in DI water suspension, and large micrometric agglomerates in skin delivery media suspension. The dimensional discrepancies among the samples tend to blur when increasing the complexity of the suspending medium. In fact, the observed primary particle size, the only size parameter often reported, do not correlate with the particle size measured in the delivery medium.

The ex vivo experiments on porcine skin indicate that the three TiO$_2$ polymorphs do not penetrate into the viable layer of skin, in agreement with previous findings.\textsuperscript{4,61} Nonetheless, all of them are able to interact with the stratum corneum, although to a different extent. Anatase and anatase/rutile are not removed with washing (μ-XRF mapping; SEM-EDS), induce a decrease in the intermolecular order of lipids (Raman spectroscopy), and strongly perturb the intercellular arrangement of the lipid bilayer in the stratum corneum to a higher extent than rutile (DSC). On the other hand, rutile is not only efficiently removed from the SC by simple washing, but produces a negligible (Raman) and a lower (DSC) alteration of the intermolecular order of SC structural molecules. The different behavior of rutile with respect to anatase and anatase/rutile in their interaction with SC may be explained in terms of both electrostatic forces and chemical oxidative potential. It has to be noted that rutile and anatase differ in surface acidity,\textsuperscript{33} which determines the surface charge at a given pH. Under the adopted experimental conditions, the surface of rutile is negatively charged, while both anatase and anatase/rutile are slightly positive. This is likely due to the stronger acidity of the titanol moieties on the rutile surface with respect to anatase and anatase/rutile, as indicated by the shift of the points of zero-charge (present work) and by their different affinity toward water.\textsuperscript{32} The net surface charge seems to govern the interaction of nano-TiO$_2$ with stratum corneum, with negative rutile being easily removed from the negatively charged skin surface.\textsuperscript{62} At the opposite, electrostatic interactions are likely to occur between the positively charged nano-TiO$_2$ (anatase and anatase/rutile) and the negatively charged corneocytes of the stratum
In turn, they determine a stronger interaction of the nano-TiO$_2$ with skin and prevent the complete removal upon washing, as illustrated by $\mu$-XRF mapping and SEM. Such finding are consistent with a parallel study conducted on a set of negatively charged, coated TiO$_2$ nanometric powders. The measured chemical oxidative potential is also consistent with the major modifications induced on the SC structure by anatase and anatase/rutile with respect to rutile. In fact, cell-free reactivity tests (oxidative reactivity and peroxidation of lipids) indicate that anatase and anatase/rutile nano-TiO$_2$ are more reactive than rutile. Such reactivity accounts for the ability of anatase and anatase/rutile to promote structural rearrangement of lipid chains, as evidenced by Raman and DSC, and suggests a TiO$_2$-induced oxidative mechanism that may alter the structure of the stratum corneum, even under indoor illumination. Both the higher oxidative potential and the stronger adhesion with the stratum corneum of anatase and anatase/rutile TiO$_2$ may explain the stronger disorganization induced by these two samples and signal a possible increase in skin permeability. Enhanced permeability may in turn allow diffusion of unwanted chemicals or nanoparticles to viable layers of the skin and possibly pose a concern for human health.

5. **Conclusions**

When contacted ex vivo with skin, positively charged anatase and anatase/rutile nano-TiO$_2$, but not negatively charged rutile, are strongly held and able to promote a structural rearrangement of the lipid bilayer in the stratum corneum. The reactivity observed in solution suggests a ROS-mediated chemical oxidative damage of lipids and organic molecules with anatase and anatase/rutile being stronger oxidative agents than rutile. Low amount of UV light ($< 1$ mW/m$^2$) is sufficient to photoactivate the ROS production in nano-TiO$_2$ and induced oxidative damage on skin exposed. The present findings strongly encourage the use of the less reactive, negatively charged rutile to produce safer TiO$_2$-based cosmetic and pharmaceutical products.
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ABBREVIATIONS

BHT  butyl hydroxyl toluene
DI   de-ionized
DSC  differential scanning calorimetry
DMPO 5,5-dimethyl-1-pyrroline N-oxide
DMSO dimethyl sulfoxide
MDA malonyldialdehyde
PZC  point of zero charge
SC   stratum corneum
TBA  thiobarbituric acid assay
µ-XRF micro X-ray fluorescent spectroscopy
XRPD X-ray powder diffraction

SUPPORTING INFORMATION

Supporting Information Available: Raman spectroscopy of nanometric TiO₂ powders and SEM-EDS analysis of anatase-contacted skin. This information is available free of charge via the Internet at http://pubs.acs.org.
REFERENCES


