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(Article begins on next page)

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Indoor illumination: a possible pitfall in toxicological assessment of photo-active nanomaterials

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Abstract

Standardization of the experimental protocols used in the hazard assessment of nanomaterials (NMs) is strongly required to reduce inconsistency among data deriving by different laboratories. The parameters that are known to modify the toxic response of cells to NMs are in fact higher than for soluble toxicants. Among them illumination, that may induce activation of some semiconducting NMs, has been poorly investigated.

The present study, conducted within the FP7 EU project SETNanoMetro, has been designed to assess the effect of indoor illumination on the oxidative potential and dispersion degree of nano-TiO₂. The generation of Reactive Oxygen Species (ROS) by four nanometric anatase or rutile-

anatase TiO₂ specimens under ordinary laboratory illumination has been evaluated by means of Electronic Paramagnetic Resonance (EPR) spectroscopy, while their ability to damage DNA has been measured by agarose gel electrophoresis using plasmid DNA as model. The effect of illumination on nanoparticles dispersion has been evaluated by Dynamic Light Scattering (DLS). The results show the occurrence of photo-activation of TiO₂ under indoor illumination that leads to the generation of ROS and slight plasmid DNA damage. Furthermore, significant differences in the amount of ROS generated were found for small variation of the intensity of the illumination. A small effect on the size distribution of TiO₂ agglomerates in water was observed.

The present findings suggest that illumination should be included among the parameters that have to be controlled during toxicological assessment of photo-active nanomaterials.

Keywords

Nanomaterials; reactive oxygen species; particle dispersion; toxicological testing; illumination.

1. Introduction

The knowledge of the hazard is a fundamental pre-requisite to reduce the risk associated to the exposure to chemicals. The current European regulation (REACH), places responsibility on industry to provide safety information on the substances. As consequence, test method standardization for hazard assessment is strongly needed [1, 2]. In the case of nanomaterials (NMs) standardization is a particularly relevant issue. Numerous specific and non-specific factors have been shown to influence the results of toxicological testing [3], such as: i) the degree of dispersion of the NM, that varies depending upon the media used [4,5,6], ii) the real dose, that, oppositely to molecular substances may not correspond to the nominal one [7,8], iii) the presence of contaminants in the materials like bacterial lipopolysaccharides (LPS) [9] or metal ions [10] iv) the occurrence of artefacts due to adsorption of reagents or intrinsic

absorbance/fluorescence of the material [3,11,12]. Much less explored is the effect of illumination during the preparation of samples and NM exposure in toxicological testing. Except in the case in which a specific illumination is necessary due to the kind of endpoint evaluated, incubation of cells in *in vitro* tests is performed in the dark. On the other hand, the preparation of the NMs and the administration to cells is performed in laboratories illuminated by artificial or natural light. During these steps, photo-activation of semiconductors materials like ZnO, CeO₂, NiO or TiO₂ may occur since indoor natural illumination and some artificial light (e.g. halogen lamps) contains UV radiation.

Among them titanium dioxide (TiO₂) and TiO₂-based materials are the most widespread NMs [13,14], being used for several purposes, e.g. as UV blockers in sunscreens and plastics [15, 16]. TiO₂ is a powerful photo-catalyst. When illuminated with UV light it generates at its surface a high amount of reactive species, a property that finds application in several fields, like in water and air remediation [17] or in the production of self-cleaning coatings and textiles [18]. The adsorption of photons with energies higher or equal to the TiO₂ band gap (>3.2 eV for anatase) results in electrons to be excited in the conduction band (e^-_{CB}) leading to the formation of a positive hole in the valence band (h^+_{VB}). These charge carriers can recombine each other or migrate at the surface where they react with electron donors or acceptors that diffuse close to the surface [19]. For example, by reacting with water and oxygen, hydroxyl radicals ($HO^\bullet$), superoxide radicals ($O_2^{\bullet-}$), singlet oxygen (1O_2), hydroperoxyl radicals ($^\bullet OOH$) and hydrogen peroxide (H_2O_2) are formed. The generation of such oxygenated radicals and molecules, commonly called Reactive Oxygen Species (ROS), affects the ability of TiO₂ to interact with cells by increasing its oxidative potential, i.e. the ability to induce an oxidative burst [20, 21]. The role of particle-derived ROS in the photo-toxicity of TiO₂ is well established [22, 23]. On the other hand, the toxicity of non-illuminated TiO₂ is not expected to be related to them [24]. Nevertheless, several studies performed in the absence of specific illumination reported TiO₂-induced effects related to the occurrence of oxidative burst [25, 26]. Whether it

is a consequence of ROS generated by light-activated TiO₂ or of cell-derived ROS is not clear since in most of the studies the illumination condition used during NMs handling is not described.

Another well-known property of TiO₂ is the superhydrophilicity: under irradiation with UV light the abundance of hydrophilic groups at the surface of TiO₂ (Ti-OH) increases, an effect that is reversed in the dark [27]. Superhydrophilicity may affect the agglomeration degree of TiO₂ suspensions in water. In fact, particle agglomeration may occur in colloidal suspensions when particles exhibit a low osmotic repulsion. In this case attractive van der Waals forces and entropy driven surface dehydration prevail leading to agglomeration [28]. This largely depends on the thickness of the Stern layer around particles [29] that in turn depends upon particles surface chemistry, in particular the abundance and type of charged groups.

Indeed, a light-induced disaggregation of TiO₂ nanoparticles under UV light was previously reported [30].

The present study is aimed to assess the effect of indoor illumination on the oxidative potential and dispersion degree of nano-TiO₂. Four samples of fully characterized nano-TiO₂ in the anatase or anatase-rutile forms, the most photo-active ones, has been selected and analyzed for their ability to generate ROS in different illumination conditions by using a set of EPR-based tests previously proposed as integrated protocol for the assessment of biological-relevant photo-activity of TiO₂ [31]. The ability to damage DNA was also tested. Finally, the effect on the NM dispersion, evaluated by means of Dynamic Light Scattering (DLS) analysis was investigated.

2. Methods

2.1 TiO₂ samples. Four types of titania NMs were considered, three commercial materials (i.e. P25 by Evonik Industries, Germany; SX001 by Solaronix, Swizerland; and PC105 by Cristal, Saudi Arabia) and one lab-made TiO₂ prepared via hydrothermal synthesis, and then coded as UT001. Details of preparation, structural and morphological characterization of the specimens

are in ref. [32]. In brief, UT001 was obtained by forced hydrolysis of an aqueous solution of $\text{Ti}(\text{TeoaH})_2$ complex (Teoa = triethanolamine; initial pH 10), carried out by hydrothermal treatment at 453 K for 90 h. Before the use, each material was suspended in water and then processed according to the following procedure in order to remove organic and inorganic impurities adsorbed onto TiO_2 NPs: i) dialysis against ultrapure water (MilliQ, Millipore) using a Spectra/Por dialysis membrane tubing (MWCO 8-12 kD or MWCO 12-14 kD); final pH of the permeated liquid in the 5-6 range, Cl^- and $\text{SO}_4^{2-} < 1$ ppm (by ion chromatography); ii) freeze-drying; iii) re-suspension in milli-Q water; iv) irradiation for 48 hours of the suspension in contact with air, added of 10 ml of H_2O_2 (30%), under UV light using a medium pressure mercury lamp (emission max at 360 nm), ca. 50 W/m^2 in the range 290-400 nm; followed by dialysis and free-drying as steps i) and ii). Step iv) ensures a complete photo-degradation of organic impurities adsorbed onto TiO_2 NPs that can change their surface properties and reactivity of TiO_2 . H_2O_2 is used as electron scavenger to speed up impurities degradation.

2.2 Surface Area Measurements. The specific surface area (SSA_{BET}) of the powders was measured by adsorption of N_2 at 77 K, applying the BET model for the analysis of results.

2.3 X-ray diffraction (XRD). X-ray diffraction (XRD) pattern of the powders were recorded with an Analytical X'Pert Pro equipped with an X'Celerator detector powder diffractometer using Cu K α radiation generated at 40 kV and 40 mA. The instrument was configured with $1/2^\circ$ divergence and receiving slits. A quartz sample holder was used. The 2θ range was from 20° to 80° with a step size ($^\circ 2\theta$) of 0.05 and a counting time of 3 s.

2.4 Morphological Characterization. TEM images were obtained with a Jeol 3010 instrument, operated at 300 kV. For the observation, powders were contacted in dry form with standard Cu grids coated with a lacey carbon film, and then introduced in the microscope. To evaluate the presence of aggregates the samples were also analyzed by Dynamic Light Scattering in 200 mM ammonia solution, in order to maximize the particles electrostatic repulsion, after 30 min sonication and adjusting the concentration depending on the sample

characteristics. The Dynamic Light Scattering system used was an ALV (Langen Germany), NIBS model (non invasive backscattering) with fixed scattering angle (173°). Through the Stokes-Einstein equation the hydrodynamic radius r_H of the agglomerates/aggregates were obtained.

2.5 Diffuse reflectance UV-Vis spectroscopy. The optical behaviour of the powders in the UV-Vis range was investigated by electronic absorption spectroscopy in the diffuse reflectance mode. Spectra were acquired with a Cary 5000 instrument (Varian), equipped with an integrating sphere coated with Spectalon[®], also used as reference. In order to avoid side effects due to differences in particle packing, the powder cell provided by Varian was used, allowing pressing a sample toward the optically pure quartz window constituting the front part of the cell. Proper amounts of powders were used, resulting in layers of ca. 3 mm in thickness, thus reaching the usual condition for correct measurements in the diffuse reflectance mode [33].

2.6 Illumination conditions and light irradiance measurements.

The irradiance in the visible and UV regions was measured with a photo-radiometer (Delta Ohm S. r. L., Padova, Italy) under natural indoor light (windows closed) and artificial illumination (halogen lamp). For the sake of comparison, the irradiance of outdoor natural light was also measured (Table 1). Measurements were made twice a day for one week in the month of September 2016 (Latitude: $45^\circ 04' 13''$ N Longitude: $7^\circ 41' 12''$ E). No UVC radiation is expected to be present in solar light and therefore was not measured.

Experiments were performed under reduced illumination (shielded light) set up under a hood by shielding the glasses, or under standardized light obtained by using a 500 W Hg/Xe lamp (Oriol Instruments) equipped with an IR water filter to avoid the overheating of the suspensions with or without a 400 nm cut-off filter. The presence of the 400 nm cut-off filter leads a radiation that contains a fraction of UVA/B of intensity intermediate between outdoor and indoor (Table 1). Finally experiments were also performed in a dark room with a red led as unique source of

light. In this case, each sample was weighted and then kept in the dark room for 24h before experiments, in order to exclude any activation of the powders.

Table 1. Light irradiance at the different illumination conditions

Light source	Light irradiance (W/m ²)			
	Visible 1050-400 nm	UVA 400-315 nm	UVB 315-280 nm	Total UVA/UVB (calculated)
Hg/Xe lamp	795 ± 73	432 ± 60	836 ± 78	1268
Outdoor	315 ± 119	3.30 ± 1.19	0.28 ± 0.13	3.6
Halogen lamp	7.30 ± 1.50	63.9x10 ⁻³ ± 5.0x10 ⁻³	7.9x10 ⁻³ ± 1.0x10 ⁻³	71.8x10 ⁻³
Natural indoor light	3.30 ± 1.40	25.6x10 ⁻³ ± 8.0x10 ⁻³	0.97 x10 ⁻³ ± 0.3x10 ⁻³	26.6x10 ⁻³
Hg/Xe lamp + filter	560 ± 58	0.164 ± 0.040	3.5 x10 ⁻³ ± 3.0 x10 ⁻³	0.168
Shielded light	0.20 ± 0.06	0.70x10 ⁻³ ± 1.0x10 ⁻³	0.75 x10 ⁻³ ± 0.07	1.4x10 ⁻³
Dark	18.7x10 ⁻³ ± 0.30x10 ⁻³	0.10x10 ⁻³ ± 0.08x10 ⁻³	0.60x10 ⁻³ ± 0.2x10 ⁻³	0.7x10 ⁻³

2.7 Generation of free radicals and singlet oxygen. All experiments were performed in ultrapure MilliQ water (Millipore, Billerica, MA). Amounts of powders corresponding the same exposed surface area (1.4 m²) calculated on the basis of the SSA_{BET} was used for all experiments (P25 25mg; PC105 16mg; UT001 30mg; SX001 15mg). The powders were transferred in a 2.5 ml quartz vial and generation of the different ROS was monitored by adding the following solutions:

1) Total reactivity: 2 ml of a 50μM solution of TEMPONE-H (1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine, Enzo Life Sciences Inc., Farmingdale, New York, US) in water;

2) Oxidative reactivity: 0.5 ml of a solution of DMPO (5,5-Dimethyl-1-pyrroline-N-oxide, Enzo Life Sciences Inc., Farmingdale, New York, US) 88 mM, and sodium formate 1M in phosphate buffer saline (pH 7.5, 0.005M);

3) Singlet oxygen generation: 2 ml of a 50 mM solution of 4-oxo-TMP (2,2,6,6-tetramethyl-4-piperidone, Sigma-Aldrich, Saint Louis, Missouri, US) in phosphate buffer saline (pH 7.4, 0.01M)

The suspensions were exposed to the different illumination conditions for 60 or 40 minutes and the generation of radical species monitored by Electron Spin Resonance (EPR) spectroscopy (Miniscope 100 EPR spectrometer, Magnettech, Berlin, Germany) on aliquots of the suspensions withdrawn with a glass capillary each 10 minutes.

Instrument settings: microwave power 7 mW, modulation amplitude 1G, scan time 80s, two scans. The negative controls were, in all experiments, the solutions illuminated in the same conditions as the samples. All experiments were repeated at least three times.

The amount of radical generated was evaluated by building a calibration curve with the stable free radicals 4-oxo-TEMPO (or TEMPONE, 4-oxo-2,2,6,6-tetramethylpiperidine-1-oxyl, Enzo Life Sciences Inc., Farmingdale, New York, US) in water in the concentrations range 50 - 0.12 μ M.

2.8 Generation of hydrogen peroxide. An amount of powder corresponding to an exposed surface area of 1.4 m² was suspended in 2 ml of water in a quartz vial and exposed to the chosen illumination conditions. The powder was removed by filtration (cellulose acetate, 0.20 μ m). The concentration of hydrogen peroxide on the supernatant was evaluated by using the method reported by Mottola et al. [34]. 50 mg of leucocrystal violet (LCV) was dissolved in 80 ml of 0.5%(v/v) HCl and diluted to 100 ml with the same solution. A buffer solution was made by mixing equal volume of 2M sodium acetate and 2M of acetic acid and adjusting the pH to 4.5 with acetic acid.

1 ml of LCV solution was added to a 1 ml of the supernatant. 4 ml of buffer and 0.5 ml of peroxidase (type I from horseradish) (1 mg/mL) was added and the solution diluted to 10 ml with water. The absorbance was measured after 10 minutes at of the sample at 596 nm against a reference prepared in the same manner but with no powder (Kontron Instruments Inc., Everett, MA). The concentration of hydrogen peroxide was determined by building a calibration curve.

2.9 Direct plasmid DNA damage. Plasmids are convenient model systems to study direct DNA damage because their sizes are well defined, the quantification of their single breaks (SSBs) by gel electrophoresis is relatively easy and accurate, the chemical environment of the DNA can be precisely controlled, and there is no biological repair processes [35]. Here pYES2 plasmid DNA (Invitrogen, Italy) was used as a model. The damage was quantified in terms of single (SSB) and double (DSB) breaks in the DNA strand. Strand breaks were detected by agarose (1 %) gel electrophoresis which separates the three forms of DNA molecules, supercoiled DNA (undamaged plasmid); open circular DNA (resulting from single-strand breaks); linear DNA (a product of double-strand breaks). Experiments were performed in a quartz vial with 0.2 mg of powder suspended in 30 μ l of MilliQ water and then vortexed. To this suspension 5 μ l of DNA solution (about 50 ng/ μ l) were added and then exposed for 20 minutes to the different illumination conditions. As control, DNA was exposed to the corresponding illumination condition, for the same time in the absence of any powder in order to exclude a direct damage to this molecule. After the exposure time (20 min) the suspension was centrifuged (15000 g) and the supernatant was used for gel electrophoresis. DNA bands were stained and visualized with ethidium bromide (Promega, Italy).

Controls of the different forms of plasmid DNA were obtained by digesting the supercoiled DNA with EcoRI enzyme in the presence or in the absence of ethidium bromide [23].

2.10 Hydrodynamic diameter. The hydrodynamic diameter was evaluated by dynamic light scattering (DLS) (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, U.K., detection limits 1 nm–6 μ m) in ultrapure water or in a 0.05 wt% solution of bovine serum albumin (BSA,

Sigma-Aldrich, Saint Louis, Missouri, US) by using a dispersion protocol adapted from the EU-FP7 project NanoGenoTox deliverable 3 (<http://www.nanogenotox.eu>). Briefly, a 2.56 mg/ml stock dispersion was prepared by pre-wetting powder in 0.5 vol% ethanol (96% purity) followed by dispersion in 0.05 wt% BSA and sonicated for 35 minutes with a probe sonicator (100 W, 40% amplitude, 20 kHz, 3 mm titania probe, Sonoplus, Bandelin, Berlin, Germany). The time of sonication and the amplitude of the sonicator power were set-up to deliver a pre-determined acoustic power according to the method developed within the EU-FP7 project NANOReg. DLS analysis was started after 10 minutes of incubation in the various illumination conditions. The results are the mean of three independent measurements each consisting in consecutive 10 runs on the same vial.

3. Results

3.1 Physico-chemical characterization of the TiO₂ samples

The XRD patterns of the four selected materials show that all materials are pure anatase, except for P25 which is a mixture of anatase/rutile (Table 2 and SI).

Table 2. Main physico-chemical features of the TiO₂ samples

	Crystalline phase ^a	Impurities	Specific surface area (m ² /g) ^b	Primary particle size (nm) ^c	Hydrodynamic diameter (nm, NH ₃ 0.2 M) ^d
P25	Anatase 80% Rutile 20%	-	55	30	36
PC105	Anatase >99%	sulphates	86	23	630 (aggregates)
UT001	Anatase >99%	carbonates/ carboxylates	47	33	34
SX001	Anatase	carbonates/ carboxylates	93	19	82 (aggregates)

^a XRD; ^b BET; ^c TEM; ^d DLS

UT001 exhibits a quite regular bi-pyramidal shaped nanoparticles with regular borders [32] whereas P25, PC105 [36] and SX001 [37] are characterized by less regular profiles (Figure S1 in SI), in agreement with the presence of a significant fraction of surface terminations different from {101} previously facets (the most stable ones), as probed by IR spectroscopy of adsorbed CO [37].

The trend exhibited by the specific surface area (SSA) is in qualitative agreement with the size of primary nanoparticles (the smaller the size, the larger the SSA). Nevertheless, only UT001 and P25 nanoparticles attained a mono-dispersion when suspended in a proper aqueous medium, whereas even in the best dispersion condition attained the other two TiO₂ powders

exhibited hydrodynamic diameters larger than primary particles, indicating they are constituted by agglomerates of nanoparticles, quite huge in size for PC105 [38].

All samples appeared, as expected, opaque to UV radiation and transparent to visible light (Figure S2 in SI). The Kubelka-Munk vs. wavelength spectra exhibited the typical absorption edge due to the valence-to-conduction band transition [39]. As expected, the absorption edge of P25 (anatase:rutile $\approx$ 80:20 by weight) is located at longer wavelength with respect the pure anatase materials, because of the narrower inter-band energy gap of rutile [40]. In all cases the onset of the absorption is located in the high energy part of the visible range, due to the coupling of photon absorption with phonon emission, one of the two results of the indirect character of the inter-band transition [41,42]. Nevertheless, an additional localized absorption seems to be present in the visible range of the spectrum of PC105, which could be due to localized charged-transfer absorptions related to the presence on the surface of these TiO₂ nanoparticles of sulphate groups.

3.2 Effect of UV radiation intensity of TiO₂ photo-reactivity

Preliminary experiments conducted in normal indoor light unexpectedly showed a significant photo-activation of all powders. However, the amount of radicals generated was highly variable during the day and the seasons, because of the different intensity of the light (Table 1). The activation of the TiO₂ powders was also monitored by gradually shielding the laboratory natural light down to the minimal amount of visible light allowing to operate, and by measuring the reactivity of the powder toward sodium formate at fixed irradiance values .

Figure 1. Effect of light intensity on TiO₂ activation. EPR signals recorded after 60 minutes of incubation of A) P25; B) UT001; C) PC105; D) SX001 in a buffered solution (PBS, 0.005M, pH 7.5) containing 1M sodium formate and 88mM DMPO. The irradiance values (UVA) measured during the experiments are reported on the graphs.

The EPR signal intensity (Figure 1), that is proportional to the powder reactivity, was found largely dependent on the light irradiance for all the samples, despite the differences in the UVA radiation intensity was very low. At a value of UVA of 0.7 mW/m², correspondent to a total UVA+UVB 1.4 mW/m² (Table 1) the lowest signal intensity was obtained for all the samples. This last condition, referred here as "shielded light" can be considered the condition most widely applicable in practice. For this reason, was chosen for the subsequent experiments.

3.3 TiO₂ photo-activation in shielded light

A set of tests were used to measure the overall reactivity of TiO₂ that may induce cell damage, including all ROS species generated by TiO₂ and the oxidative/reductive processes that follow

the direct reaction of molecules with the surface charge carriers [43]. This was achieved by EPR spectroscopy using three different probes, sodium formate, the hydroxylamine TEMPONE-H and the piperidone 4-oxo-TMP. Hydrogen peroxide generation was evaluated by a spectrophotometric method as described in the Method Section. The experiments were performed under the shielded light condition described above and the data compared with those obtained by using the filtered Hg/Xe lamp (positive control) or in the dark (negative control) . In Figure 2 the amount of radicals generated in the three illumination conditions, at the last time point considered in the kinetic, are reported. The full kinetics of generation are reported in the SI. A substantial reactivity toward the three probes was observed following irradiation with the filtered Hg/Xe lamp, while no hydrogen peroxide generation was detected in any conditions (not reported). Some differences among samples was observed: UT001 and SX001 were the most reactive toward sodium formate (Figures 2 and S5 in SI), UT001 and P25 appeared the most reactive toward TEMPONE-H (Figures 2 and S4 in SI), while the most active in generating singlet oxygen was SX001 (Figures 2 and S6 in SI). An increase of the amount of ROS generated with time was observed for all samples in all tests (SI).

Figure 2. ROS generation by the TiO₂ samples in different illumination conditions. A) radicals generated in the presence of sodium formate (oxidative reactivity) ; B) radicals generated in the presence of TEMPONE-H (total reactivity); C) generation of singlet oxygen. The data are expressed as amount of radicals generated per unit surface area of the powder, at the last point considered in the kinetic (see SI). Illumination conditions are indicated in each panel.

The amount of ROS generated in the shielded light condition was negligible when measured with sodium formate or 4-oxo-TMP. However, the samples, and in particular UT001 and P25, was still active toward TEMPONE-H, albeit the amount of ROS generated was one order of magnitude lower than those generated with the filtered Hg/Xe lamp. Differently to what previously reported [44], in the dark a negligible reactivity was observed. This was because in the present case the powders were kept for 24h in the dark, thus suggesting a possible role of pre-illumination.

3.4 TiO₂-induced damage to plasmid DNA in shielded light

The reactivity was evaluated by incubating the powders with SC-pDNA in the various illumination conditions and by measuring the DNA damage by agarose electrophoresis (Figure 3). The induction of DNA strand breaks was evaluated by the conversion of the supercoiled form (SC) to open circular (OC) and linear (L) forms.

Figure 3. Effect of illumination on TiO₂ -induced damage to double stranded supercoiled plasmid DNA. The plasmid DNA was exposed for 20 min to the TiO₂ samples in different illumination conditions (irr: filtered Hg/Xe lamp, sl: shielded light, d: dark). Damage was evaluated as capability to induce the formation of, open circular DNA (OC) and linear DNA(L) from native supercoiled double stranded DNA (SC). (M) marker; (DNA irr) DNA irradiated by the filtered Hg/Xe lamp without powders; (DNA EcoRI) DNA digested with EcoRI enzyme; (DNA EcoRI+EtBr) DNA digested with EcoRI enzyme + ethidium bromide.

As expected, when illuminated with the filtered Hg/Xe lamp, all the TiO₂ samples caused a clear damage to DNA: in fact, an increase in the intensity ratio between the bands correspondent to the open circular (OC) plasmid DNA and supercoiled double stranded DNA (SC) was observed for all samples with respect to plasmid DNA irradiated in absence of powder and the DNA treated with the powders in the dark. This effect is a clearly consequence of a ROS dependent or independent oxidative damage directly induced by activated TiO₂. No bands correspondent to the linear form (L) of DNA was observed. When exposed to shielded light, a

very slight increase of the OC/SC intensity band ratio was observed with all samples except than for PC105.

3.5 Light-induced aggregation/disaggregation

To evaluate the possible effect of illumination on the agglomerate size distribution of TiO₂ UT001 was chosen. This sample was the best candidate for DLS analysis since it is composed by particles having a narrow size distribution. The mean hydrodynamic diameter of the powder and the polydispersity index (PDI) were measured in both water and in a 0.05% bovine serum albumin (BSA) solution after sonication following a standardized protocol as described in the method section. Measurements were performed after exposing the suspension for 10 minutes in the dark, indoor light and filtered Hg/Xe lamp. A further condition, i.e. illuminating with the full range of visible, UVA and UVB radiations (Hg/Xe lamp without filter) was also used to induce the highest activation possible of the powders.

Figure 4. Effect of illumination on TiO₂ dispersions. Panels A and B: hydrodynamic diameters of UT001 in A) water and B) 0.05% BSA solution, under different illumination conditions: dark (a), indoor light (b), filtered Hg/Xe lamp (c), Hg/Xe lamp (d). Panel C: Z average values (bars) and the polydispersion indexes (PDI) (points) of UT001 in water (black) and in BSA solution (gray).

In water, the powders appeared organized in agglomerated (Figure 4). No significant variations in mean hydrodynamic diameter were found depending by illumination, except for a moderated shift when the suspension was irradiated with the Hg/Xe lamp. However, a significant decrease of the PDI was observed in all illumination conditions suggesting that disaggregation occurred at some extent. When dispersed in the 0.05% BSA the TiO₂ suspension appeared more uniformly dispersed (lower polydispersion index) in the dark than in water. This was expected since proteins can act as surfactants by adsorbing at the surface and increasing the repulsion among particles. Still, the particles appeared agglomerated, with a small fraction of monodisperse particles. When illuminated, a shift of the mean hydrodynamic diameter toward higher diameters was unexpectedly observed. This effect was particularly relevant after illumination with the UV/vis light. In this conditions, the Z-average value was three time higher than those observed in the dark.

4. Discussion

The data presented herein demonstrate that, in spite of the very low amount of UV light in normal indoor natural illumination ($15.94 \pm 4.8 \text{ J/m}^2$ during 10 minutes), photo-activation of TiO₂ occurs, leading possible photo-induced effects on cells during toxicological testing. Since photo-activation is largely dependent upon the intensity of UVA/B radiation, illumination may be considered a possible a source of variability of the toxicological data obtained in different laboratories.

4.1 Identification of the ROS generated in shielded indoor illumination.

TiO₂ is able to generate several different ROS. Photo-generated electrons may reduce oxygen to superoxide radicals (equation 1) while holes oxidize water leading to the generation of the highly reactive hydroxyl radicals (equation 2).

Hydroxyl and superoxide radicals may further react to generate secondary species that are hydroperoxyl radicals, the conjugated acid of the superoxide anion (equation 3), and hydrogen peroxide (equation 4). Hydrogen peroxide may further react with conduction band electrons generating hydroxyl radicals (equation 5).

Singlet oxygen (¹O₂) is also generated by a mechanism still under discussion [45, 46].

Formate ions are sensitive probes for the evaluation of the oxidative reactivity of TiO₂ [47], since they are able to react with both photo-generated holes and hydroxyl radicals to form carboxylate radicals, but not with superoxide radicals. Oppositely, TEMPONE-H measures the total reactivity of TiO₂, being able to react with all species, including superoxide radicals, forming the stable radical species TEMPONE.

In the shielded light condition used, the reactivity of the powders toward sodium formate was very low (Figure 5A). At the same time no singlet oxygen (Figure 5C) or H₂O₂ generation (data not shown) was observed. On the other hand, small amount of TEMPONE radicals were detected for SX001 and UT001 in the presence of TEMPONE-H (Figure 5B), likely generated following reaction of the probe with superoxide radicals. The ability of TiO₂ to stabilize superoxide radicals by coordination with Ti⁴⁺ ions exposed at the surface [48] may account for their presence in the reaction system. Alternatively, the higher reactivity of TiO₂ toward

TEMPONE-H by respect to formate may be due to the scavenging of the photo-generated electrons by the probe that inhibits the recombination of the charge carriers [49].

The occurrence of photo-activation suggests the possible capability of the powders to induce oxidative damage also in the presence of very low amount of light. Among the various possible targets of oxidative damage DNA is the most relevant under a toxicological point of view, since it may be related to cell death, mutation or cancer. Nucleic acids are particularly sensible to ROS [50] that may induce nucleosides oxidation, inter-strand cross-links and strand breaks formation [51]. The ROS most involved are hydroxyl radicals and singlet oxygen, while superoxide radicals are known to be inert toward biomolecules [51]. However, they generate other reactive species through reactions 3-5 or in biological environment through Haber-Weiss cycle [52] and therefore it may indirectly induce DNA damage.

The tendency of the molecules to get close enough to the surface to react with both short-living ROS species or directly with charge carriers depends upon several factors like diffusion rate and ability to bind to it. For this reason, it is not possible to directly transfer the reactivity of TiO₂ toward the probes used in EPR experiments to biomolecules. Therefore, the powders were further tested for their ability to induce strand breaks to DNA by a direct mechanism (not mediated by cells stimulation) using supercoiled plasmid double-stranded DNA (SC-pDNA) as model (Figure 3). In shielded light, a very small increase in the intensity ratio between the band correspondent to the open circular plasmid DNA and supercoiled double stranded DNA was observed for all samples except than for PC105. However, this effect was low if compared with those observed with the positive control (filtered Hg/Xe lamp), and likely irrelevant in cells, where natural antioxidant systems are present. On the other hand, we cannot exclude the generation of punctual defects like oxidized nucleosides or cross-links. Further experiments will be necessary to evaluate the relevance of the present findings in cells.

4.2 Variability among anatase samples

The analyzed samples are all uncoated and characterized by the anatase form, the most reactive one. However, they exhibit significant differences in physico-chemical features (Table 2) that may reproduce the variability encountered among the TiO₂ NM commercially available.

Albeit all samples are characterized by particles having a predominance of exposed {101} facets and exhibiting higher energy terminations, in the case of UT001 facets are quite regular, whilst other high energy surface terminations, i.e. exposing Ti and O sites with a high coordinative unsaturation level, are present on SX001 [37]. Both UT001 and SX001 have carbonate and carboxylate contaminants, mainly in the bulk, while SX001 has sulphate groups as surface contaminants. The samples differ also for the morphology: UT001 and P25 are composed by single particles, PC105 is actually in the form of quite large aggregates while SX001 is constituted by small aggregates of primary particles. Finally, P25 contains also rutile nanoparticles.

Focusing on the optical properties of these materials, differences in surface texture and the occurrence of a limited agglomeration seemed do not result in significant difference in the absorption spectra of UT001 and SX001. Conversely, sulphation and large aggregation appeared related to an enhanced absorption of PC105 in the high energy visible range. Finally, as expected, the presence of rutile in P25 resulted in a shift of the inter-band transition edge toward longer wavelength [39].

The ability to generate ROS by the four samples examined was quantitatively and qualitatively similar. However, some differences were observed. In particular, UT001 and SX001 appeared overall more reactive on a surface area unit basis by respect to the other two samples, in the test carried out using TEMPONE-H under shielded illumination conditions (Figure 2). Such differences are not due to adsorption in the visible range, since the only samples having a small adsorption out of the UV range is PC105, but likely to the presence in the bulk and at the surface of carbonate/carboxylate species [36].

The effect on plasmid DNA was similar for all samples except for PC105 that exhibited a lower reactivity, in agreement with the lower capability to generate ROS. These data suggest that variability in term of photo-activity may be found depending upon small variation in morphological and surface properties that may be further amplified by the different surface area of the TiO₂ samples.

4.3 Effect of illumination on TiO₂ dispersions.

As discussed in the introduction, illumination was previously reported to affect the agglomeration degree of TiO₂ in water [30]. This is a very important issue since the size of agglomerates is known to modulate both the real dose experienced by cells, due to differences in sedimentation rates, and the cell uptake [7]. In the present case, little changes in mean size were observed in all illumination conditions when the powders were dispersed in water (figure 4); however the size distribution of aggregates appeared narrower when illuminated than in the dark, as suggested by the lower polydispersion index, indicating that the photo-activation may affect the dispersion degree of TiO₂ powders. On the other hand, when a protein was added to the system, a clear agglomeration was observed at the more extreme illumination conditions. This effect is relevant since it may occur in cell media, where proteins are generally added as nutrients for cells. To elucidate the reasons of this behaviour is out of the scope of this report and will be the object of further investigation. However, we may speculate that the photo-activation induces conformational changes to the absorbed protein that in turn leads to particle agglomeration.

5. Conclusions

In conclusion, the data presented herein indicate that the intensity of illumination during sample preparation and exposure is an important parameter in *in vitro* testing of photo-active semiconducting NMs. It therefore has to be controlled and accurately reported. In fact, the lack of this information may limit the inter-laboratory comparability of toxicological data.

Albeit operating in a dark room appears to be the best condition during NMs handling, based on our results for pure anatase or anatase-rutile samples a shielded light, correspondent to a maximum of total UV irradiance of 1.4 mW/m^2 , may be suggested to minimize the effects related to the photo-activation of the samples. Cellular studies are in progress to validate this conclusion. Note however, that the present finding apply only to NMs having optical properties similar to TiO_2 , and not to all photo-active NM, like for example the forms of TiO_2 purposely designed to absorb in the visible region by doping or adsorption of dyes.

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Supplementary information

Indoor illumination: a possible pitfall in toxicological assessment of photoactive nanomaterials.

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Figure S1. Morphology of the samples. HRTEM micrographs of the four selected materials: (A) UT001, (B) P25, (C) SX001 and (D) PC105. Scale bar in panels = 20 nm.

Figure S2. Adsorption spectra. DR UV-Vis spectra of: A) P25; B) UT001, C) PC105; D) SX001 in air. The Y axis is limited to 1.0, the limit of a correct application of the Kubelka-Munk function (Körtum, 1969).

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Figure S3. XRD patterns of the samples

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Figure S4. Reactivity of TiO₂ samples toward TEMPONE-H in different illumination conditions: A) filtered Hg/Xe lamp; B) reduced illumination C) dark. Panels on the left: generation of TEMPONE radicals by the TiO₂ samples (● UT001, ○ SX001, □ P25, ▲ PC105, ■ no powder) when in contact with a solution of TEMPONE-H (50 μM). The data are expressed as amount of radicals generated per unit surface area of the powder. Panels on the right: representative EPR spectra recorded after 40 minutes. (a) UT001, (b) SX001, (c) P25, (d) PC105, (e) no powder. The amount of radicals is proportional to the intensity of the signal.

Figure S5. Reactivity of TiO₂ samples toward sodium formate in different illumination conditions: A) filtered Hg/Xe lamp; B) reduced illumination C) dark. Panels on the left: generation of carboxylate radicals by the TiO₂ samples (● UT001, ○ SX001, □ P25, ▲ PC105, ■ no powder) in contact with a solution (0.005M PBS, pH 7.4, 88mM DMPO) of sodium formate (1M). The data are expressed as amount of radicals generated per unit surface area of the powder; Panels on the right: representative spectra recorded after 60 minutes (a) UT001, (b) SX001, (c) P25, (d) PC105, (e) no powder. The amount of radicals is proportional to the intensity of the signal.

Figure S6. Generation of singlet oxygen by TiO₂ samples. Panel on the left: amount of TEMPONE radicals generated by the TiO₂ samples (● UT001, ○ SX001, □ P25, ▲ PC105, ■ no powder) in a solution of 4-oxo-TMP (50mM) in phosphate buffer (pH 7.4, 0.01M) when illuminated with the filtered Hg/Xe lamp. Panel on the right: representative spectra recorded after 60 minutes (a) UT001, (b) SX001, (c) P25, (d) PC105, (e) no powder. The amount of radicals is proportional to the intensity of the signal.

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Table S1. Hyperfine splitting constants of the radical species detected

Radical specie	Solvent	Hyperfine constants	splitting
DMPO/CO ₂ · ⁻	0.005 mM phosphate buffer saline pH 7.4	a _H 15.4 G; a _N 18.5 G	
TEMPONE	water	a _N 15.78 G	
TEMPONE	0.01 mM phosphate buffer saline, pH 7.4	a _N 15.75 G	

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