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The noncellular reduction of MTT tetrazolium salt by TiO_2 nanoparticles and its implications for cytotoxicity assays

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ABSTRACT

We report results of noncellular tests, revealing the occurrence of photocatalytic interactions between titanium dioxide (TiO_2, titania) nanoparticles and the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide] cytotoxicity indicator. These interactions induce the reduction of MTT and formation of purple formazan under biologically relevant conditions. Classical MTT assays have been performed to evaluate the production of formazan in DMEM-F12 and RPMI-1640 cell culture media (containing 10% fetal bovine serum-FBS) treated with Degussa-P25 TiO_2 nanoparticles, in the absence of cells. The colorimetric determinations revealed the noncellular MTT to formazan transformation induced by TiO_2 nanoparticles, under conditions commonly used for *in vitro* cytotoxicity testing of nanomaterials. The formazan precipitation was found to be proportional to the TiO_2 concentration, being enhanced under laboratory daylight exposure. The photocatalytic nature of the studied effect was assessed under UV irradiation at 365 nm. The biological significance of the reported reaction was established with respect to cellular reference experiments performed on V79-4, HeLa and B16 cell lines. The results show false viability increases with up to 14% (for TiO_2 concentrations generally higher than 50 μg/ml), induced by the TiO_2–MTT reaction. This type of artifacts may lead to underestimated toxicity or false proliferation results.

1. Introduction

In vitro toxicology of nanomaterials (NMs) represents today a developing field of research, aiming towards the identification and understanding of the potential health hazards associated to engineered and naturally occurring nanoparticles (NPs) (Arora et al., 2012). Among the commonly studied NM, titanium dioxide (TiO_2, titania) is of particular importance due to its wide utilization in food, pharmaceutical and beauty care industries as well as in antimicrobial and self cleaning technologies (Kwon et al., 2008).

In numerous *in vitro* studies involving titania nanoparticles (Iavicoli et al., 2011), the primary evaluation of TiO_2 toxicity is based on the MTT viability assay (Wang et al., 2007; Jin et al., 2008; Zhu et al., 2009; Soto et al., 2006; Han et al., 2008; Sayes et al., 2006; Wadhwa et al., 2011). In the presence of living cells, the MTT tetrazolium salt [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] is enzimatically reduced to a crystalline water-insoluble purple/blue formazan compound, the amount of which is proportional to the number of viable cells in the tested samples. To quantify cell viability, the produced formazan is solubilized in specific solvents (dymethil sulfoxide (DMSO), isopropanol), the colored solution being afterwards analyzed spectrophotometrically at wavelengths usually between 450 and 600 nm, depending on factors such as the solvent used and pH (Twentyman and Luscombe, 1987; Plumb et al., 1989). The measured optical absorbance is thus proportional to the amount of dissolved formazan and the number of viable cells. The obtained results are analyzed with respect to appropriate control samples.

Although the MTT to formazan transformation is not yet fully understood it is generally accepted that it involves the cleavage of MTT in the presence of an appropriate electron-coupling reagent (Altman, 1976; Nineham, 1955).

On the other hand, TiO_2 is a semiconductor material known to possess photocatalytic properties (Linsebigler et al., 1995; Carp et al., 2004; Chen and Mao, 2007; Fujishima et al., 2000; Hashimoto et al., 2005; Kumar and Devi, 2011). In this context, photocatalysis refers to the ability of titania nanomaterials to catalyze redox reactions of molecules adsorbed on their surface, when exposed to light of specific wavelengths ($\lambda < 385$ nm for TiO_2). In aqueous environments, these photocatalytic processes may take place by either direct charge transfer (of electrons (e^-) and holes (h^+)

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photogenerated at the TiO_2 surface) or intermediated by reactive oxygen species (ROS) such as hydroxyl radicals ($^{\cdot}OH$) or superoxide anions ($^{\cdot}O_2^-$) formed at the TiO_2–water interface (Konstantinou and Albanis, 2004; Hoffmann et al., 1995; Kang et al., 2001). In this context, the question naturally arises whether TiO_2 could induce the MTT to formazan transformation under conditions relevant for *in vitro* biological studies. If so, these effects could lead to false conclusions on the toxicity of TiO_2 nanomaterials.

Here we report results clearly indicating the occurrence of noncellular TiO_2–MTT interactions leading to the formation of purple/blue formazan. For our study we have used the commercial Degussa P25 titania (EVONIK Industries AG, Essen, Germany), a nanomaterial frequently used in reported cytotoxicity studies (Han et al., 2008; Sayes et al., 2006; Wadhwa et al., 2011). A throughout physicochemical characterization of precisely the TiO_2 used in the present study was recently reported by Popescu et al. (2013). Briefly, the used P25 titania consists of a mixture of anatase and rutile polymorphs, with an anatase/rutile weight ratio of 85:15(%). It shows average crystallite sizes of 30 nm for anatase and 50 nm for rutile and a Brunauer–Emmet–Teller (BET) specific area of 49 m^2/g (Popescu et al., 2013). The TiO_2 nanoparticles have been used in the present study without any further modification or functionalization.

The occurrence of cell mediated interferences between TiO_2 and MTT has been previously addressed in a cellular study conducted by Wang et al., these authors concluding that MTT reduction may be induced by $^{\cdot}O_2^-$ anions overproduced in cells exposed to the action of TiO_2 nanoparticles (Wang et al., 2011). We show however that the reduction of MTT takes place irrespective of the presence of cells, being a characteristic of its interaction with titania nanoparticles. This report aims only to reveal the occurrence of this interaction in noncellular experiments under biologically relevant conditions and to point out its photocatalytic nature. A systematic investigation of the mechanisms by which this reaction takes place and how it depends on the illumination and titania nanomaterials properties, makes the subject of a study in progress in our laboratory.

2. Materials and methods

2.1. Noncellular experiments (MTT–TiO_2 reaction)

Multiple sets of samples consisting of equal volumes (1 ml) of DMEM-F12 or RPMI-1640 culture media (10% FBS), containing 2.5, 7.5, 15, 25, 50, 75, 100, 150, 200, 250 and 300 μg/ml P25 titania, have been placed in 24-well culture plates. The plates were thermostat incubated (TI) for either 24 or 48 h at 37 °C, in a 5% CO_2 and 90% relative humidity (RH) atmosphere. Following incubation, the culture media have been discarded and volumes of 300 μl MTT solution (1 mg/ml MTT in phosphate buffered saline (PBS)) have been added to each well. Identical sets of samples have been afterwards kept for 2 h (MTT phase) under either thermostat conditions (case A) or room atmospheric conditions (RACs). The RAC experiments (aimed to reveal the influence of light upon the studied samples) underwent either in the dark (case B) or exposed to laboratory natural daylight illumination (case C). After 2 h, the MTT solution was removed from all samples and 300 μl DMSO were added to solubilize the eventually formed formazan crystals. Volumes of 100 μl have been afterwards transferred to 96-well plates (care being taken to avoid the inclusion of suspended TiO_2 nanoparticles). Optical absorbance has been determined at 540 nm using a Multiskan EX (Thermo Scientific) spectrophotometer. Two types of controls have been used in each experiment: negative controls (NCs) consisting of 1 ml of untreated culture media and positive controls (PCs) consisting of series of samples treated with the mentioned TiO_2 concentrations in which, instead of MTT, only PBS (MTT solubilization agent) was added, according to the above mentioned procedure. The NCs indicate whether the effect was due to the presence of TiO_2 nanoparticles while PCs take into account possible opacity effects induced by the incomplete sedimentation of the suspended nanoparticles.

To test the photocatalytic nature of the TiO_2–MTT reaction, MTT samples containing 300, 250 and 200 μg/ml TiO_2 have been prepared by dispersing the appropriate amounts of titania in volumes of 2 ml of MTT solution (1 mg/ml MTT in phosphate buffered saline (PBS)). The samples, accompanied by an appropriate MTT control (pure MTT solution), have been placed in 300–900 nm transparent cuvettes and exposed to UV light (365 nm) for 30 min. The cuvettes' contents were afterwards transferred to 2 ml tubes (Eppendorf) and centrifuged (12.000 rpm, 10 minutes) to separate titania and the formed formazan. The supernatant (excess MTT) was removed and volumes of 2 ml DMSO were added the tubes being vortexed to solubilize the formazan. The tubes were afterwards centrifuged again and the formazan solution was collected. The optical densities (ODs) of samples and controls were measured and the solutions were placed again in cuvettes, photo shoots being taken with a Canon EOS Rebel XSi camera.

2.2. Cellular significance experiments

Viability determinations, aimed as biological significance reference for the noncellular results obtained in case A, have been performed on three cell lines – V79-4 (ATCC® CCL-93™, normal fibroblasts from Hamster Chinese lung), HeLa (ATCC® CCL-2™, human cervix carcinoma) and B16-F10 (ATCC® CRL-6475™, mouse melanoma) – using the above described MTT assay. The viability of the cells used in all experiments exceeded 95% (Trypan Blue exclusion method).

The cells were seeded in 1 ml of DMEM-F12 culture medium containing 10% FBS (complete culture medium), at a density of 1×10^5 cells/cm^2, in 24-well culture plates. After 24 h, the culture medium (DMEM-F12 + FBS) was discarded and replaced with fresh complete medium containing dispersed P25 TiO_2 at the concentrations specified in the noncellular experimental section. All cellular MTT assays have been performed following precisely the protocol described for the noncellular (case A) experiments, but in the presence of cells.

The significance of the noncellular results has been determined by comparing the means of viabilities obtained from optical densities measured in cellular systems OD_i^{cells}, with those calculated based on corrected optical densities (OD_i^{new}), given by $OD_i^{new} = OD_i^{cells} - OD_{noncell}$, with $OD_{noncell} = \frac{1}{N}\sum_{i=1}^{N} OD_i^{noncell}$, N being the number of measurements ($N = 5$ in our case) and i the measurement index taking values $i = 1,2,3,...N$. The two viabilities are thus given by $V_{cells} = \frac{1}{N}\sum_{i=1}^{N} V_i^{cells} = \frac{1}{N}\sum_{i=1}^{N}\left(\frac{OD_i^{cells}}{OD_{control}^{cells}} \times 100\right)$ and $V_{new} = \frac{1}{N}\sum_{i=1}^{N} V_i^{new} = \frac{1}{N}\sum_{i=1}^{N}\left(\frac{OD_i^{new}}{OD_{control}^{new}} \times 100\right)$ where $OD_{control}^{cells}$ represents the optical density of the control used in the cellular experiment (untreated cells) and $OD_{control}^{new}$ was obtained from $OD_{control}^{new} = OD_{control}^{cells} - OD_{control}^{noncell}$, $OD_{control}^{noncell}$ being the control of the noncellular experiment (case A).

All MTT tests have been performed using five identical samplings for each TiO_2 concentration.

3. Statistical analysis of results

The statistical significance of the obtained data was assessed using the SigmaPlot-11 statistics suite, by means of one-way ANOVA (when data normality and variance equality allowed the test) or one-way ANOVA on ranks (when one of the two conditions was not