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Research paper

Photocatalytic Fe-doped n-TiO_2: From synthesis to utilization of *in vitro* cell models for screening human and environmental nanosafety

Teresa Balbi[a], Valentina Caratto[b], Rita Fabbri[a], Giulia Camisassi[a], Silvia Villa[b], Maurizio Ferretti[b], Laura Canesi[a,*]

[a] Department of Earth, Environmental and Life Sciences-DISTAV, University of Genoa, Corso Europa 26, 16132 Genoa, Italy
[b] Department of Chemistry and Industrial Chemistry-DICCI, University of Genoa, Via Dodecaneso 31, 16146 Genoa, Italy

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ABSTRACT

The utilization of different types nanomaterials (NMs) in environmental remediation and wastewater treatment requires information on the potential harmful effects on human and environmental health. In this light, the utilization of human cell models together with cells from lower organisms, representative of different environmental compartments, could represent a valuable tool for the *in vitro* screening of the potential toxicity of different NMs used in nanoremediation. Among NMs, n-TiO_2, because of its peculiar optical and chemical properties, is widely applied for photosensitized UV oxidation of organic pollutants. Moreover, development in design of metal- and non metal- doped TiO_2 with extended photocatalytic activity in the visible region represents the subject of ongoing research.

In this work, the cytotoxic effects of three different types of recently synthetized Fe-doped n-TiO_2 were compared in two cell models widely utilized for screening cellular toxicity of NMs in humans and aquatic organisms, human vascular endothelial cells (HECV) and immune cells (hemocytes) of the marine invertebrate, the mussel *Mytilus spp*, respectively. Parallel studies were carried out using N-doped n-TiO_2. The results indicate both distinct and common behavior (agglomeration state) in different media (human cell culture medium and mussel hemolymph serum) and biological effects (cytotoxicity, nitric oxide production) of different types of doped- n-TiO_2 in different cell models. Although *in vitro* studies represent a first step in the toxicological assessment of NMs, studies comparing their effects on human and aquatic invertebrate cells that take into account the effects of different exposure media represent an useful tool for evaluating potential cytotoxicity of those NMs, like TiO_2-based photocatalytic NMs, widely applied in environmental remediation, and whose potential risks are poorly understood.

1. Introduction

Nanotechnology is still seen by society as an emerging and almost unknown technology; in this light, any use of nanomaterials (NMs) involving their direct introduction into the environment in the absence of information on their potential harmful effects for humans and other organisms might prevent their widespread application. This in particular applies to the use of nanotechnology as a tool to protect the environment through pollution prevention, treatment, and cleanup [1, 2]. Despite the wide scientific production on nanoremediation (mainly for ground and freshwater water and soils), there are few contributions on the potential hazard of these NMs for human/environmental health [1–4]. Therefore, among urgent research needs and priorities it is of critical importance to incorporate nanosafety into the development of novel nanotechnologies and products -safety by design- for environmental applications. In this light, the utilization of cell models of both mammalian species and lower organisms, representative of different environmental compartments, could represent a valuable tool for the *in vitro* screening of the potential toxicity of different NMs used in nanoremediation. In particular, aquatic invertebrates have been shown to represent sensitive models for evaluating ecotoxicity of different types of NMs [5–7].

A variety of NMs like metal and non metal oxides, carbon nanotubes, dendrimers, polymers, etc. are increasingly being used in air, water, and soil remediation [1–4]. Within the class of inorganic NMs, semiconducting photocatalytic nano-oxides, such as ti-

* Corresponding author.
E-mail address: laura.canesi@unige.it (L. Canesi).

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tanium dioxide nanoparticles (n-TiO_2) have gained considerable interdisciplinary attention and research interest because of their diverse potentials in energy and environmental applications. Their major advantage is the capacity to chemically convert pollutants into nonhazardous products, through oxidation and reduction under light irradiation as external energy source. Photocatalytic n-TiO_2 have the widest range of applications in environmental remediation, wastewater treatment, recovery of precious metal via TiO_2-assisted reduction, organic synthesis, photokilling activity, or self-cleaning activity [8–11]. When n-TiO_2 is irradiated by UV light, electrons are promoted across the band gap (3.2 and 3.0 eV) in the anatase and rutile polymorphs, respectively [3] into the conduction band, leaving holes in the valence band. These holes are highly oxidizing and react with the adsorbed hydroxide ions, inducing the formation of oxidizing species, hydroxyl radicals in particular [8, 12]. The applications of n-TiO_2 are limited by its requirements for UV activation: consequently, considerable research efforts are being directed towards expanding the optical response of n-TiO_2 to the visible light region through a variety of modification strategies, in particular the incorporation (doping) of transition metals, lanthanides and non metals into TiO_2 [13–15]. Due to the potential widespread applications of doped n-TiO_2 in water and wastewater treatment and remediation, its possible impact on human health and on aquatic biota represents a serious concern.

Different mammalian models are used for evaluating the toxicity of undoped n-TiO_2 at the cellular level [3, 16]. Similarly, undoped n-TiO_2 has been shown to exert different effects also in the cells of marine invertebrates (reviewed in [7]). In these models, different exposure media were utilized: culture medium containing variable percentages of serum (mainly foetal calf serum FCS) for mammalian cells, and artificial sea water (ASW) or standard culture media added with suitable proportions of NaCl for marine invertebrate cells [7]. However, there is increasing awareness that in mammalian cells, the biological effects of NMs, including photocalalytic n-TiO_2, are due not only to their physico-chemical properties (core composition, size, shape, surface characteristics) but also to their behavior in biological fluids and interactions with serum protein components [17, 18]. Interactions of NMs with invertebrate serum proteins have been recently demonstrated also in invertebrates (earthworms and marine mussels) [19, 20]. These data underline how evaluation of cytotoxicity of NMs in standard *in vitro* assays using different model systems would require some basal information on particle behavior in different testing media.

Different types metal and non-metal doped n-TiO_2 with photocatalytic activity in the visible spectrum have been recently synthetized by members of our group [21, 22]. In this work, the toxicity of three types of Fe-doped n-TiO_2 was compared in human and marine invertebrate cells. Human vascular endothelial cells (HECV) are widely utilized for evaluating human toxicity of NMs, including TiO_2 [16, 23]. The hemocytes of the marine mussel *Mytilus spp*, that represent the circulating cells responsible for innate immunity and resemble in structure and function mammalian macrophages, have proven an useful model for screening the environmental toxicity of NMs [24–27]. The effects of different types of Fe-doped n-TiO_2 were evaluated by standard cytotoxicity test for each cell model. Moreover, production of nitric oxide (NO), a signaling molecule that represents a common mediator of inflammatory processes in both mammalian and invertebrate cells [28] was evaluated. Particle behavior (agglomeration state) in different exposure media (human cell culture medium-HCEM and mussel hemolymph serum-HS, respectively) was evaluated. The results were compared with those obtained using N- doped n-TiO_2 and undoped n-TiO_2 synthetized in the same experimental conditions [21,22].

2. Methods

2.1. Synthesis and characterization of Fe-doped n-TiO_2

Three different n-TiO_2 samples (Sample 1, 2, 3) doped with 0.5% (theoretical molar ratio) of Fe^{3+} were synthesized by sol–gel process using titanium tetraisopropoxide as the precursor of titania, 2-propanol as solvent, iron(III) chloride as dopant source through calcination at 350 °C (for details see [21]). Samples were characterized by means of X-ray powder diffraction, diffuse reflectance spectroscopy, scanning electron microscopy, Brunauer–Emmett–Teller analysis. All samples showed the typical structure of anatase plus an amount of brookite of 26.5 and 27.8 wt% in Samples 1 and 2, respectively, and 35.5 wt% in sample 3 [21]. Photocatalytic activity of Fe-doped TiO_2 nanopowders was evaluated under simulated solar light irradiation as Methylene blue degradation according to ISO standard 10678:2010 [21]. For comparison, N-doped n-TiO_2, as well as undoped n-TiO_2 synthetized by sol-gel methods in the same experimental conditions, were utilized [22].

2.2. Particle behavior in exposure media

Particle size (Z-average and polydispersity index, PDI) was determined by DLS (Malvern instruments LTD), using a Zetasizer software, version 7.11. Measurements were performed in triplicate samples, each containing from 10 to 14 runs for determining Z-average. The samples, obtained diluting the particle suspension in milliQ water in human cell culture medium or mussel hemolymph serum to a final concentration of 0.63 mg/mL, were dispersed with an Omni Sonic Ruptor Ultrasonic Homogenizer using the microprocessing tip (5/3200) at a power of 30% for 3 min before DLS analyses.

2.3. Human cell assays

Human umbilical vein endothelial cells (HECV), purchased from Interlab Cell Line Collection (ICLC, Genova, Italy), were grown in Dulbecco's modified Eagle's medium (DMEM) (Euroclone, Milan, Italy) supplemented with 10% Foetal Calf Serum - FCS (Euroclone, Milan, Italy) at 37 °C in 5% CO_2 humid incubator. Cells were seeded in 96-well plates (10^4 cells/well) and incubated under visible light for 24 h at 37 °C in 0.2 mL DMEM without phenol red (exposure medium) supplemented with 10% FCS. Cells were then incubated for 24 h with different concentrations of the three different samples (1, 2 and 3) (0.1–1–10 µg/mL) suitably diluted from a 0.63 mg/mL stock solution in exposure medium supplemented with 0.4% FCS (Human cell exposure medium – HCEM, pH 7.3, ionic strength 150 mM) and dispersed by bath sonication for 15 min. Control samples were run in parallel. For each sample, four experiments were performed in six replicates. Cell viability was evaluated by the 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [21]. For comparison, the effects of the highest concentration tested (10 µg/mL) of Sample 1–3 on cell viability was also determined in RAW 264.7 mouse macrophages. Cell culture conditions and assays were the same as those utilized for HECV cells.

Readings were corrected for background absorbance of NM suspensions in exposure medium in the absence of cells [29]. Nitric oxide (NO) production in HECV cells was also evaluated by the Griess reaction in cells exposed for 24 h to Sample 2 (0.1–1- and 10 µg/mL) [21].

2.4. Marine invertebrate cell assay

Mussels (*Mytilus galloprovincialis* Lam.) 4–5 cm long, sampled from an unpolluted area at Cattolica (RN) were obtained from SEA

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Table 1
Characterization of different Samples of Fe-doped n-TiO_2 (Samples 1–3) (from Caratto et al., 2016 [21]), N-doped n-TiO_2 (Sample 4) and undoped n-TiO_2 (Sample 5) (from Caratto et al., 2012 [22]) synthetized by sol-gel method. Photocatalytic activity was evaluated as % MB degradation (see Methods). DLS analysis was performed in particle suspensions in milliQ water (2 mg/ml).

Sample	Reagent ratio (Ti-isopropoxide/ 2-propanol/ H_2O)	BET (m^2/g)	Energy gap (eV)	Photocatalytic activity	DLS (nm)
1	7.8: 16.7: 1	16.73	3.22	43 ± 3.3	854 ± 32
2	1: 12: 1	106.15	3.23	77 ± 2.5	722 ± 164
3	1: 2: 5	112.15	3.22	75 ± 3.0	570 ± 30
4	1: 2: 5	97.82	3.00	96 ± 4.0	593 ± 20
5	1: 2: 5	120.08	3.64	97 ± 2.5	560 ± 30

(Gabicce Mare, PU) and kept for 1–3 days in static tanks containing artificial sea water (ASW) (1 l/mussel) at 16 °C. Sea water was changed daily. Hemolymph was extracted from the posterior adductor muscle of 8–20 mussels, using a sterile 1 mL syringe with a 18 G1/2″ needle. With the needle removed, hemolymph was filtered through a sterile gauze (0.22 µm) and pooled in 50 mL Falcon tubes at 4 °C. Hemolymph serum (HS, pH 7.3, ionic strength 500 mM) was filter sterilized and obtained as previously described [20]. Hemocyte monolayers, prepared as previously described [27], were incubated for 30 min at 16 °C under visible light with different concentrations of the three different Samples (1, 2 and 3) (0.1–1–10 µg/mL) suitably diluted from a 0.63 mg/mL stock solution in filter sterilized hemolymph serum (HS) and dispersed by bath sonication for 15 min. Lysosomal membrane stability (LMS) in control hemocytes and hemocytes pre-incubated with different samples was evaluated by the Neutral Red (NR) Retention time assay [20, 27]. Hemocyte monolayers on glass slides were incubated with 30 µL of a NR solution (final concentration 40 µg/mL from a stock solution of NR 40 mg/mL DMSO); after 15 min excess dye was washed out, 30 µL of ASW, and slides were sealed with a coverslip. Every 15 min slides were examined under an optical microscope and the percentage of cells showing loss of the dye from lysosomes in each field was evaluated. For each time point 10 fields were randomly observed, each containing 8–10 cells. The endpoint of the assay was defined as the time at which 50% of the cells showed sign of lysosomal leaking (the cytosol becoming red and the cells rounded). For each experiment, control hemocyte samples were run in parallel. Triplicate preparations were made for each sample. All incubations were carried out at 16 °C.

Hemocytes were also exposed to 2 h to different concentrations of Sample 2 (0.1–1- and 10 µg/mL) and NO production was evaluated by measuring nitrite accumulation by the Griess reaction as previously described [27].

3. Results and discussion

3.1. Particle characterization and agglomeration in different aqueous media

Three different n-TiO_2 samples doped with 0.5% (theoretical molar ratio) of Fe^{3+} were synthetized by sol-gel methods [21] and their physico-chemical characteristics are summarized in Table 1. The reagents ratio (titanium tetraisopropoxide/ volume solvent (2-propanol-water)) influenced surface area (BET analysis) and aggregation state (DLS). In particular, a high solvent volume with respect to the titanium tetraisopropoxide concentration is needed to observe the formation of nanoparticles with high surface area and similar dimension of agglomerates. The quantity of titanium tetraisopropoxide with respect to the solvent (water and isopropanol) is important parameter for the TiO_2-Fe synthesis; in fact, the too low amount of solvent inhibits the correct formation of the gel, inducing the agglomeration of the nanoparticles. Energy gap analysis did not show differences in the absorption spectra, a result consistent with the same iron doping concentration (0.5%) utilized for all syntheses. Photocatalytic activity, evaluated under solar-simulated light irradiation, was higher for Sample 2 and 3, in line with higher surface areas measured by BET.

The three samples were made of anatase containing a proportion of brookite from 26.5 to 35.5 wt% [21]. The photocatalytic activity of the anatase-brookite composites depends on the relative percentages of the two phases. However, the behavior of the various anatase-brookite composites also depends on many other factors such as specific surface area, crystallinity degree, crystallite sizes of the different phases, surface hydroxylation and preparation method [30]. Although sample 3 had a higher brookite content, in all samples values were within the optimum range of brookite (20–40%) for photolytic activity in anatase-brookite composites. In this light, the lower photolytic activity of Sample 3 could be mainly ascribed to its lower surface area than to its crystalline composition.

DLS analysis of suspensions of different samples was performed first in milliQ water, providing average diameters of 854 ± 32, 721.9 ± 164 and 569.9 ± 29.9 nm, respectively, for Samples 1, 2 and 3. The obtained values of PI (polydispersity index) were high, ranging between 0.3 and 0.8, approximately, indicating that the particles are very polydisperse. For comparison, the physico-chemical characteristics of N-doped n-TiO_2 (Sample 4) and of undoped n-TiO_2 (Sample 5) synthetized by sol-gel methods in the same experimental condition are also summarized in Table 1 [22].

To investigate particle behavior in exposure media utilized for biological assays in human and marine invertebrate cells, DLS analysis was performed in Fe-doped n-TiO_2 suspensions in human cell culture exposure medium (HCEM) and mussel hemolymph serum (HS) and the results are reported in Fig. 1. In HCEM (Fig. 1A), Sample 1 formed micrometric size agglomerates, much larger (1500 nm) than in H_2O, whereas smaller agglomeration was observed for Sample 2 (approximately 500 nm). Sample 3 agglomeration was similar to that observed in H_2O (600 nm). However, as in milliQ water, PI values were high, ranging between 0.25 and 0.7.

A distinct behavior was observed in mussel HS (Fig. 1B), where Sample 1 showed smaller agglomerates than in H_2O and in HCEM (500 nm). Sample 2 agglomeration was similar to that observed in H_2O and higher than in HCEM (800 nm), whereas Sample 3 agglomerates did not differ in different media. Also in HS, high PDIs were observed (between 0.4 and 0.8).

For comparison, agglomeration of N-doped n-TiO_2 (Sample 4) and undoped n-TiO_2 (Sample 5) in different exposure media was evaluated. In both HCEM and HC, N-doped n-TiO_2 formed similar agglomerates of approximately 500 nm in size (Fig. 1A and B), with high PDIs (from 0.3 to 0.8). Similar results were obtained for undoped n-TiO_2.

Overall, the results show that in different media all Fe-doped n-TiO_2 samples were highly polydisperse, and that the type of medium affects particle behavior. The agglomeration state in aqueous media depends not only on particle characteristics, but also on several factors (such as ionic strength, pH, presence of proteins and other organic compounds), that are very different in biological fluids of mammalian and invertebrate species. In particular, in

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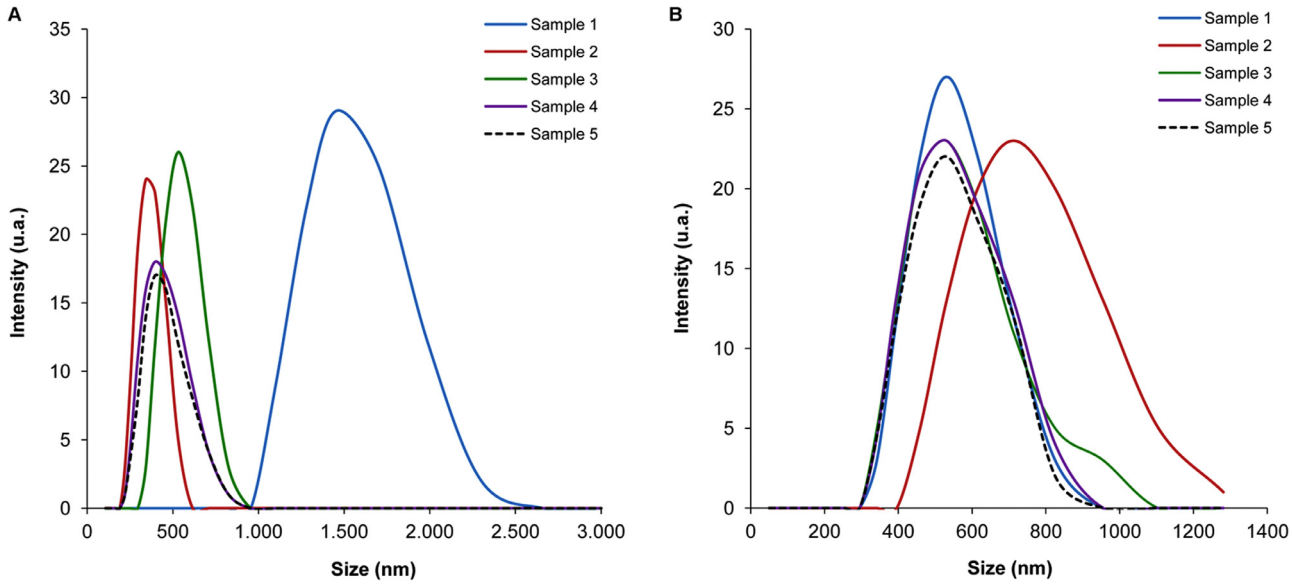

Fig. 1. Size distribution graph by intensity for suspensions of different types of Fe-doped n-TiO_2 (Samples 1, 2 and 3), N-doped n-TiO_2 (Sample 4) and undoped n-TiO_2 (Sample 5) in different exposure media, as determined by DLS Analysis (see Methods). A) human cell culture medium (HCEM); B) hemolymph serum (HS).

Mytilus, a marine osmoconformer, HS has a ionic strength similar to that of sea water and therefore much higher than that of mammalian serum. Ionic strength is one of the main factors affecting behavior of NMs, including n-TiO_2, in aqueous media, leading to higher agglomeration/sedimentation [31, 32]. However, the results obtained with Sample 1 indicate higher agglomeration in HCEM than in HS, indicating that factors other than higher ionic strength can influence particle behavior. When comparing the composition of different media in terms of organic biomolecules, mussel serum has a total protein content at least 5 times lower than mammalian culture medium containing 10% foetal bovine serum, and it is characterized by a distinct protein profile [33], as well as by a peculiar metabolic profile [34]. All these factors may contribute to different agglomeration states of Fe-doped n-TiO_2 particles in different media. Moreover, the effect of exposure media was also apparently particle-specific, since no differences in agglomeration state in HCEM and HC were observed for N-doped and undoped n-TiO_2 samples. Although the factors affecting the behavior of Fe-doped n-TiO_2 are presently unknown, it must be underlined that the concentration of nanoparticle suspension for DLS analysis is select function of polydispersity index (PDI), and data were recorded at higher particle concentrations than those utilized for biological assays. This limitation generally applies to the study of NP behavior in biological exposure media for *in vitro* cytotoxicity assays.

3.2. In vitro cell assays

The possible cytotoxic effects of different Fe-doped n-TiO_2 samples were compared in Human umbilical vein endothelial cells (HECV) and in the hemocytes of the marine invertebrate *Mytilus* spp. The times of exposure, endpoint and concentrations were chosen on the basis of previous data obtained on NP cytotoxicity in each cell model [21, 27].

Samples 1–3 have been previously tested for their cytotoxicity in HECV cells [21] and the results are summarized in Fig. 2A. Sample 1 showed lowest biological effects, inducing a small decrease in cell viability only at the highest concentration tested; large agglomerates could be seen in the extracellular medium, and no cellular uptake was observed (data not shown). These data are in line with the physico-chemical characteristics of Sample 1, that had the lowest surface area, higher agglomeration in HCEM, and showed the lowest catalytic activity and therefore low capacity of oxyradical production that may be involved in cytotoxicity. Sample 2 induced a significant decrease in cell viability only at the highest concentration tested (10 µg/ml) (−40%; $p \leq 0.05$). A similar, but clearer dose-dependent effect was induced by Sample 3. Representative images of control HECV cells and cells exposed to Sample 2 and 3 (10 µg/mL) are reported in Fig. 2B, showing the presence of both individual particles and their agglomerates in the extracellular medium, as well as cellular uptake, that were particularly evident for Sample 2.

For comparison, cytotoxicity of Samples 1–3, at the highest concentration tested, was also evaluated in RAW 247 mouse macrophages (Fig. 3). No effects on cell viability were observed (Fig. 3A). As for HECV cells, in RAW macrophages exposed to Sample 2 more abundant extracellular particles and their agglomerates, as well as cellular uptake could be observed by optical microscopy (Fig. 3B).

Mussel hemocytes were exposed to Samples 1–3 (0.1–1–10 µg/mL), as previously described with different types of commercial NPs, including n-TiO_2, and lysosomal membrane stability (LMS) was evaluated as a classical endpoint of toxicity [20, 27]. As shown in Fig. 4A, Sample 1 and 3 did not affect hemocyte LMS at any concentration tested, whereas a small but significant decrease was observed with Sample 2 at the highest concentration (−18%; $p \leq 0.05$). In Fig. 4B representative images of NR loaded hemocytes are reported, showing the presence of intact lysosomes not only in controls, but also in cells exposed to Sample 2 and 3 (10 µg/mL), respectively. No cellular uptake of either Sample 2 and 3 agglomerates could be observed. However, in hemocytes exposed to Sample 2, large agglomerates could be seen in the extracellular medium, and smaller agglomerates adhering to cellular pseudopodia (arrows). In contrast, in cells exposed to Sample 3 few agglomerates could be seen, indicating that most of them were washed out during sample preparation. Overall, the results indicate that both mussel hemocytes and mammalian macrophages show little sensitivity to the potential cytotoxicityof different samples of Fe-doped n-TiO_2, independent of particle surface area, agglomeration state in different media and photocatalytic activity. The observed lack of effects in both hemocytes and macrophages could be related to the common function of these two cell types that are responsible for innate immunity, and therefore in the cellular-

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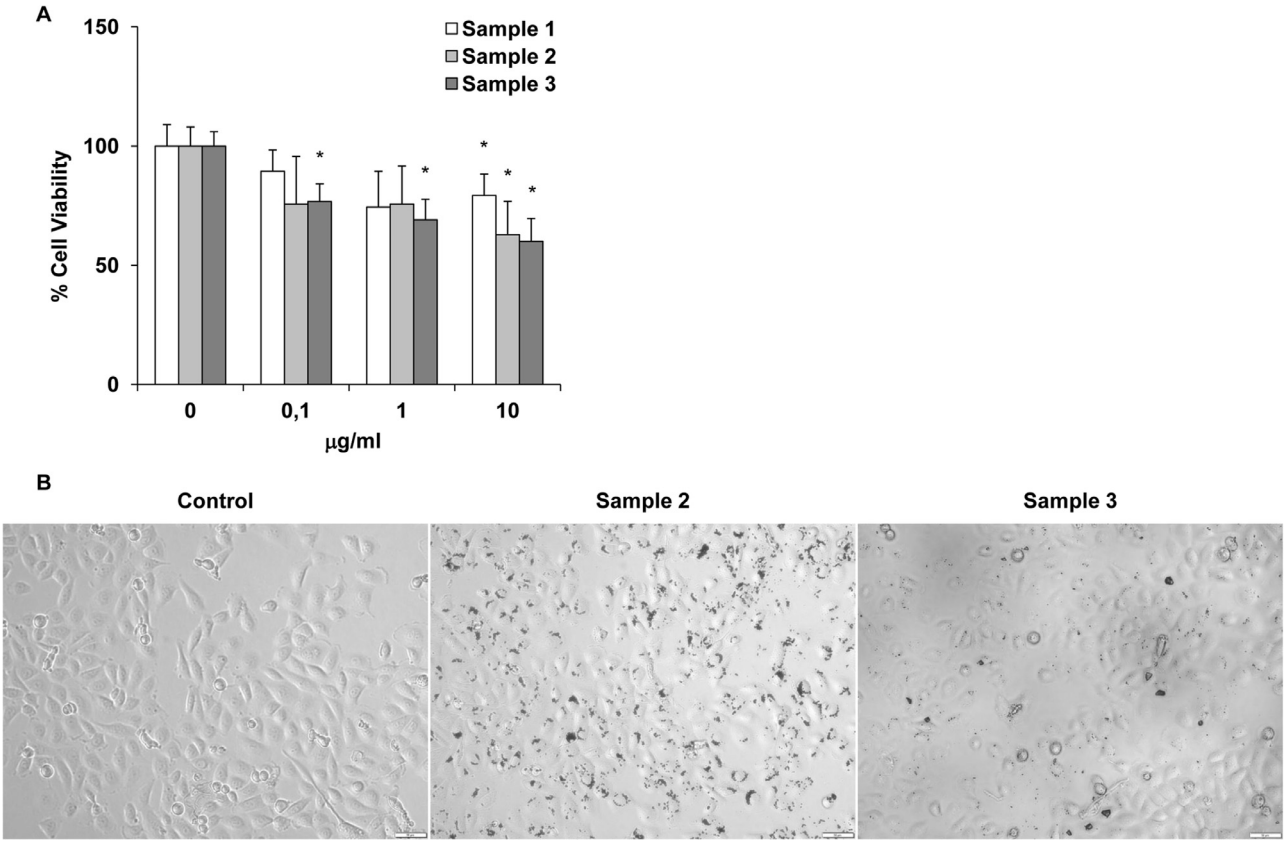

Fig. 2. Effects of different types of Fe-doped n-TiO_2 (Sample 1, 2 and 3) suspensions in HCEM on cell viability of human HECV cells, evaluated by the MTT assay (A) (modified from [21]). Data, expressed as percent of control values (untreated cells) and representing the mean ± SD of four experiments in triplicate, were analyzed by one-way ANOVA followed by Tukey's post hoc test ($^* = p \leq 0.05$). In B are reported representative optical microscopy images of untreated cells, and cells exposed to Sample 2 and 3 (10 µg/mL).

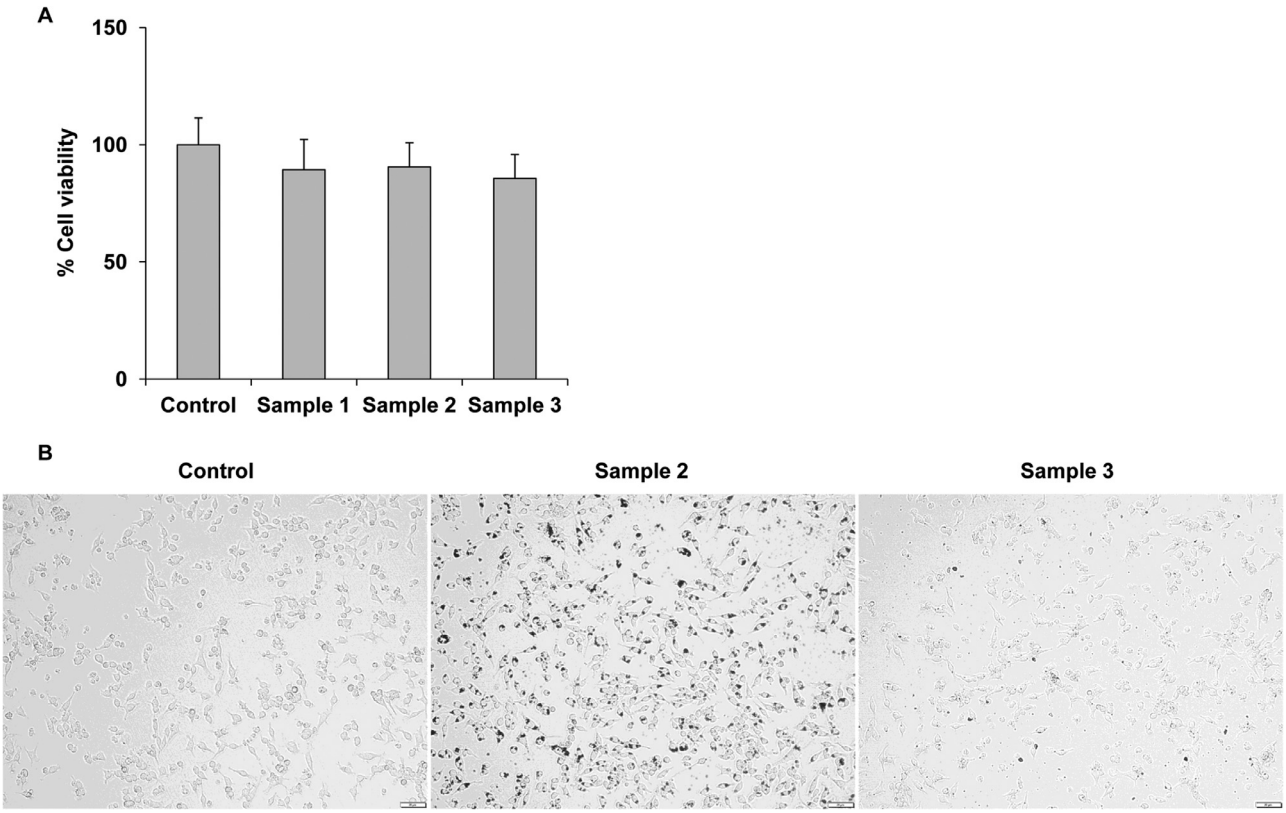

Fig. 3. Effects of different types of Fe-doped n-TiO_2 (Sample 1, 2 and 3) suspensions in HCEM (10 µg/mL) on cell viability of mouse RAW 264.7 mouse macrophages, evaluated by the MTT assay (A). Data, expressed as percent of control values (untreated cells) and representing the mean ± SD of four experiments in triplicate, were analyzed by one-way ANOVA followed by Tukey's post hoc test. In Bare reported representative optical microscopy images of untreated cells and cells exposed to Sample 2 and 3.

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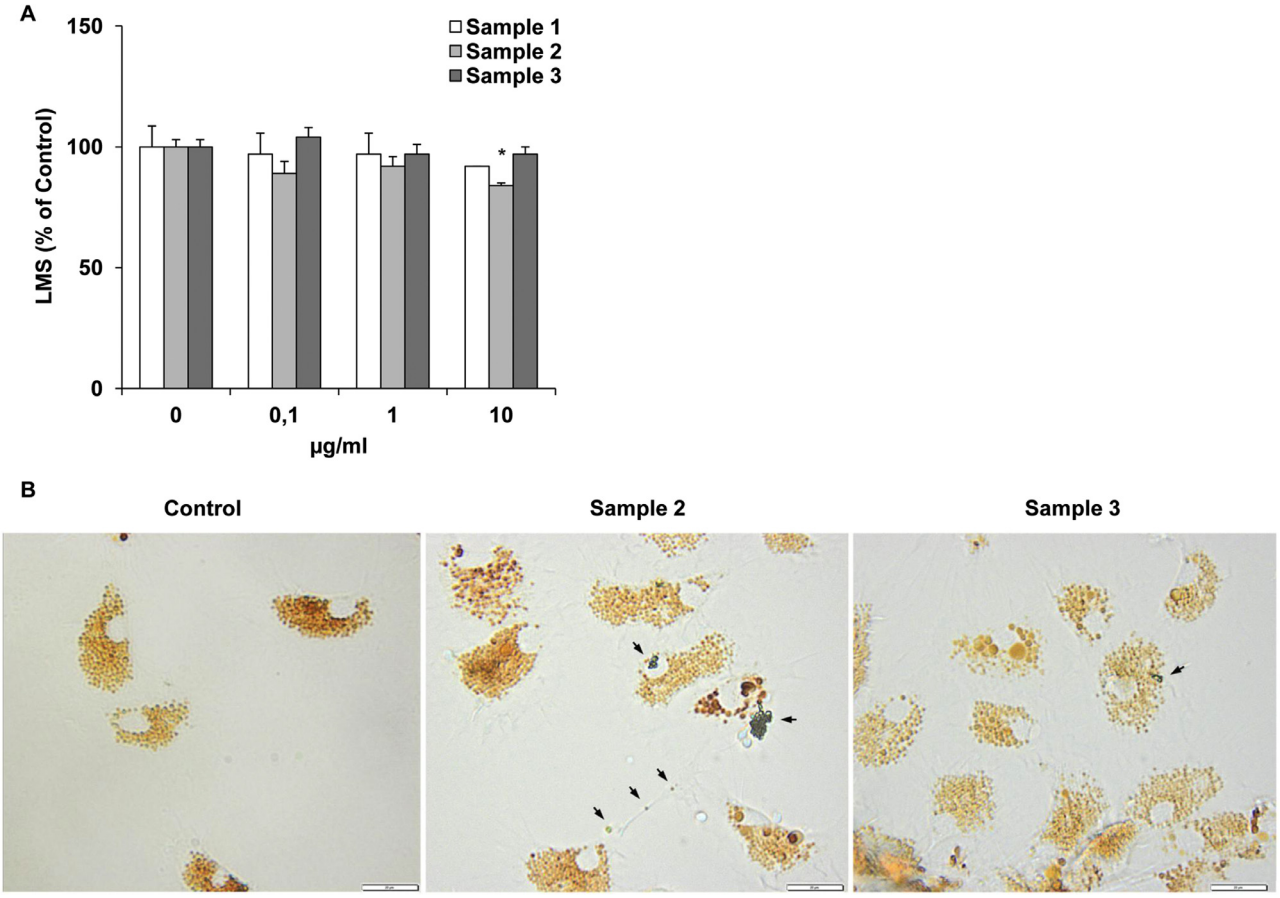

Fig. 4. Effects of different types of Fe-doped n-TiO_2 (Sample 1, 2 and 3) suspensions in HS on lysosomal membrane destabilization of *Mytilus* hemocytes, evaluated as NR retention time. Cells were incubated as described in methods with suspension of Samples 1, 2 3 (0.1, 1, 10 µg/mL) in HS. A) Data, expressed as percent of control values (untreated hemocytes) and representing the mean ± SD of four experiments in triplicate, were analyzed by one-way ANOVA followed by Tukey's post hoc test ($^* = p \leq 0.05$). B) Representative optical microscopy images of untreated cells and cells exposed to Sample 2 and 3.

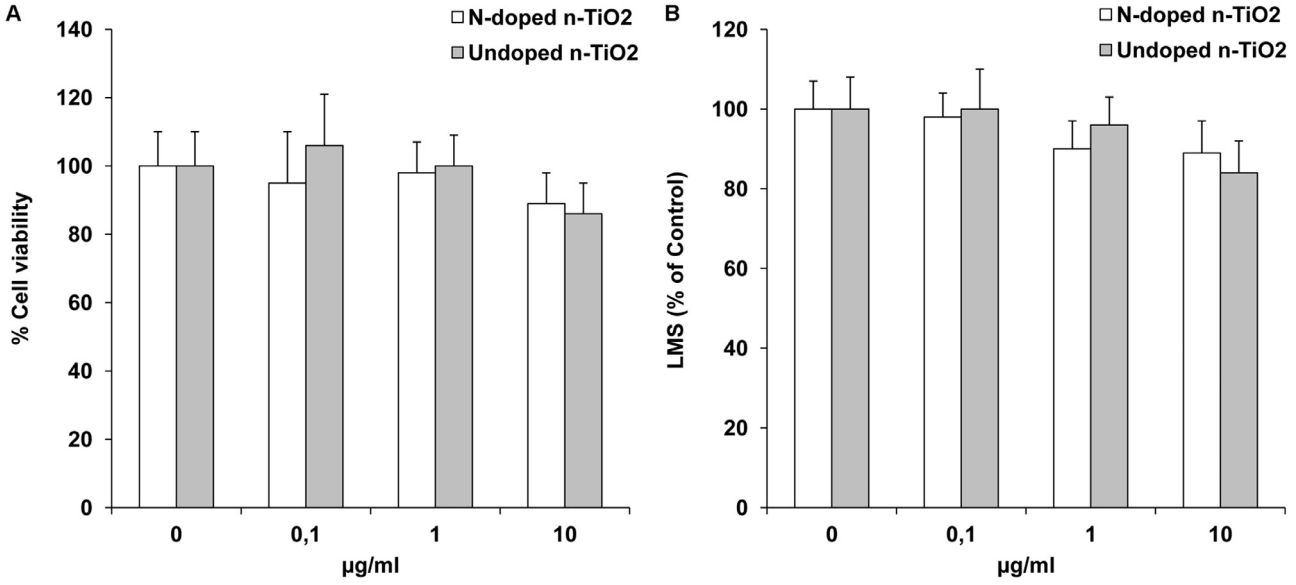

Fig. 5. Effects of N-doped and undoped n-TiO2 (Sample 4 and 5) in HECV cells (A) and *Mytilus* hemocytes (B), evaluated by the MTT assay and the NR retention time, respectively. Cells were incubated as described in methods with suspension of Samples 4 and 5 (0.1, 1, 10 µg/mL) in either HCEM or HS. Data, expressed as percent of control values (untreated cells) and representing the mean ± SD of four experiments in triplicate, were analyzed by one-way ANOVA followed by Tukey's post hoc test.

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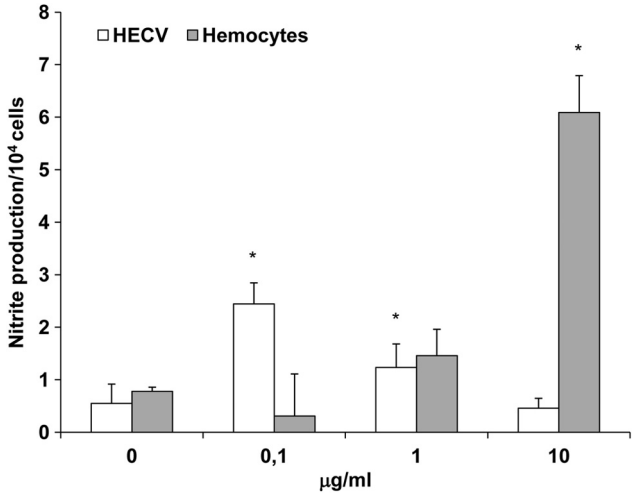

Fig. 6. Effects of different concentrations of Fe-doped n-TiO_2 (Sample 2) on nitric oxide (NO) production in HECV cells and *Mytilus* hemocytes, evaluated as nitrite production. HECV cells were exposed to Samples 2 for 24 h [18] and data are expressed as µmoles/10^4 cells. Mussel hemocytes were exposed for 2 h as previously described [26] and data are expressed as nmoles/10^4 cells. Data, representing the mean ± SD of 6 replicates, were analyzed by ANOVA followed by Tukey's post test. (* = $p \leq 0.05$).

mediated defence mechanisms against foreign materials in mussels and mammals, respectively [25, 26].

For comparison, the cytotoxic effects of N-doped and undoped n-TiO_2 were evaluated in HECV cells and mussel hemocytes. No effect on cell viability or lysosomal membrane stability were observed in either cell type (Fig. 5A and B). Whether the presence of iron, a transition metal involved in redox reactions, can play a role in the biological effects of Fe-doped n-TiO_2 is the subject of further investigation.

In both human endothelial cells and in *Mytilus* hemocytes, toxicity of n-oxides can involve different events, including increased production of oxygen and nitrogen reactive species that lead to oxidative stress [23, 27]. Among these, Nitric oxide (NO) is an intracellular signalling molecule ubiquitously found in animals involved in multiple biological functions, including the immune response to inflammatory stimuli [28]. In HECV cells, only Sample 2, at concentrations that were not cytotoxic, was shown to increase NO production, indicating the induction of pro-inflammatory processes [22]. Therefore, the effects of Sample 2 were also tested in the invertebrate cell model. In Fig. 6 data on NO production in HECV cells and mussel hemocytes after exposure to Sample 2 for 24 and 2 h, respectively, are compared. The results show that also in mussel hemocytes Sample 2 stimulated NO production, although the maximal effect was observed at the highest concentration tested (10 µg/mL). These data demonstrate that this type of Fe-doped n-TiO_2 could induce inflammatory responses in both cell models at concentrations that induced no (HECV) or low (hemocytes) cytotoxicity.

4. Conclusions

Overall, the results represent a first attempt to screen the potential *in vitro* toxicity of newly synthetized photocatalytic metal and non-metal doped n-TiO_2 using both human and marine invertebrate cell models, also considering the potential behaviour of NMs in different experimental exposure media. This approach represents a material synthesis and design strategy to compare the potential harmful effects of different types of NMs on human and environmental health. The results underline distinct particle behaviour in different media and different degrees of toxicity for different types of Fe-doped n-TiO_2 samples in different cell models. HECV cells were particularly sensitive to the potential toxicity of different Fe-doped n-TiO_2 samples, and the observed decreases in cell viability appear to be correlated with the surface area, photocatalytic activity and agglomeration state in exposure medium. This is of particular importance since, whatever the route of exposure, NMs would enter the circulatory system and inevitably would come into contact with endothelial cells, that are located at the inner surface of blood vessels, and represent the biological barrier between blood and tissues. In endothelial cells, NP toxicity can involve different events, including inflammation and oxidative stress, possibly leading to cardiovascular disease [23].

In contrast, little toxicity of different samples was observed in mussel hemocytes, independent of their agglomeration state in different exposure media. Interestingly, despite the lack of toxic effects, Sample 2 was able to stimulate NO production also in mussel hemocytes, although at the highest concentration tested. The mechanisms involved in the capacity of this type of Fe-doped n-TiO_2 to induce inflammatory processes in both cell models require further investigation.

The results of DLS analysis of different metal- and non-metal doped n-TiO_2 in mussel HS represent the first data on NM behaviour in the biological fluids of a marine invertebrate species in comparison with standard cell culture media containing mammalian serum. The results support the hypothesis that, in high ionic strength media, like marine water and biological fluids of marine invertebrates, NM behaviour and biological effects may be profoundly different with respect to those observed in freshwater media or biological fluids of mammalian and vertebrate species [7, 20]. However, in both synthetic and natural marine waters, behaviour of n-TiO_2 is influenced not only by ionic strength and pH, but also by the presence of organic material, this affecting particle distribution and biological uptake [31–32]. What is more, once within the organism, the effects of NMs at the cellular level will be mainly due to their rapid interactions with components of biological fluids, forming 'protein/biomolecule coronas' whose composition affects particle recognition by cells and consequent toxicity, as widely demonstrated in mammalian systems (reviewed in [35]). In *Mytilus* serum, we have recently identified the protein corona formed around amino modified polystyrene nanoparticles (PS-NH_2) [20]. The formation of a protein corona was shown to affect the responses of mussel hemocytes in comparison to sea water medium. These data represented the first evidence for the formation of a NP bio-corona in marine invertebrates, and underlined the importance of utilizing a physiological exposure medium during *in vitro* testing of NPs with marine invertebrate cells.

Although *in vitro* screening studies obviously represent a first step in the toxicological assessment of NMs in humans and other biological systems, the comparison between human and aquatic invertebrate cell models is potential interest for evaluating potential cytotoxicity of those NMs, like TiO_2-based photocatalytic NMs, that are applied in environmental remediation, and whose potential risks are poorly understood [36–38]. The factors and processes affecting ecotoxicity are complex, and knowledge of the potential impacts of manufactured nanoparticles on the environment and on human health is still limited. The knowledge of processes, including absorption, distribution, metabolism and excretion, as well as careful toxicological assessment is critical in order to determine the effects of NMs in different biological systems [36]. Most societal issues are based on these unknown risks of using nanoscale materials for site remediation [1]. In this light, it is of critical importance to incorporate nanosafety into the development of novel nanotechnologies and products -safe by design- for environmental applications.

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Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest.

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