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Predictive tests to evaluate oxidative potential of engineered nanomaterials

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Abstract. Oxidative stress constitutes one of the principal injury mechanisms through which particulate toxicants (asbestos, crystalline silica, hard metals) and engineered nanomaterials can induce adverse health effects. ROS may be generated indirectly by activated cells and/or directly at the surface of the material. The occurrence of these processes depends upon the type of material. Many authors have recently demonstrated that metal oxides and carbon-based nanoparticles may influence (increasing or decreasing) the generation of oxygen radicals in a cell environment. Metal oxide, such as iron oxides, crystalline silica, and titanium dioxide are able to generate free radicals via different mechanisms causing an imbalance within oxidant species. The increase of ROS species may lead to inflammatory responses and in some cases to the development of cancer. On the other hand carbon-based nanomaterials, such as fullerene, carbon nanotubes, carbon black as well as cerium dioxide are able to scavenge the free radicals generated acting as antioxidant. The high numbers of new-engineered nanomaterials, which are introduced in the market, are exponentially increasing. Therefore the definition of toxicological strategies is urgently needed. The development of acellular screening tests will make possible the reduction of the number of in vitro and in vivo tests to be performed. An integrated protocol that may be used to predict the oxidant/antioxidant potential of engineered nanoparticles will be here presented.

1. Introduction

Nanotechnology rapidly developed in recent years and a large number of new nanomaterials for a wide range of applications have been introduced in the market [1,2]. Concerns exist about possible adverse health effects following human exposure to those nanomaterials, which may release nanoparticles [3,4]. Because of a growing number of new NPs is expected to be produced in the future is urgent to define strategies to predict their possible impact on health.

Oxidative stress constitutes one of the principal injury mechanisms through which engineered NPs can induce adverse effects. Zhang and coworkers [5] recently reported the development of a quantitative structure-activity relationship (QSAR) model to predict the acute pulmonary inflammation potential of various metal oxide NPs based on the values of the conduction band energy levels. In fact, when the biological and material energetic states are similar, the permissive electron transfer could

lead to the formation of oxidizing or reducing molecules that influence the level of antioxidants and/or increase the production of ROS. However, the proposed predictive tool may be not applicable to materials other than oxides. On the other hand processes different from electron transfer between surface and cells (e.g. physical interaction with biomolecules or mechanical damage to cells) likely participate to the overall mechanism of toxicity.

The aim of this study is to get insight on the capability of three different nanomaterials to induce oxidative damage by generating ROS or by directly damaging biomolecules. Two types of metal oxides, i.e. titanium oxide and amorphous silica nanoparticles, were compared with a carbon soot sample. These samples were chosen since they are expected to be very different in surface reactivity. TiO_2 is known to be a potent photo-catalyst while amorphous silica is an inert covalent material. Carbon exhibits a reactivity that depends upon its allotropic form. An integrated protocol to evaluate the oxidative potential of the materials has been used. The capability of NPs to interfere with the ROS was evaluated by means of EPR spectroscopy/spin-trapping or probing technique while the ability to cause oxidative damage to lipids, proteins and DNA were evaluated by means of UV-Vis spectroscopy, SDS-PAGE electrophoresis and agarose gel electrophoresis, respectively.

2. Materials and methods

2.1. Samples

The rutile–anatase TiO2 (Aeroxide P25), hereafter named TiO_2, was purchased by Evonik, (Essen, Germany). Carbon soot, hereafter named C, was purchased from Sigma-Aldrich s.r.l (St. Louis, MO; lot number 390127-25G) was obtained by resistive heating of graphite. The amorphous silica (Aerosil OX 50), hereafter named SiO_2, was purchased by Degussa Frankfurt A.M., Germany.

2.2. Generation of free radicals

Free radical release was monitored by means of EPR spectroscopy (Miniscope 100 EPR spectrometer, Magnettech) using the spin trapping technique (5,5-dimethyl-pirroline-N-oxide, DMPO, as spin trapping molecule) with a procedure described in a previous study [6]. Briefly, hydroxyl radicals were detected by using DMPO (5,5-dimethyl-1-pyrroline-N-oxide, Alexis Biochemicals, San Diego, CA) in the presence of hydrogen peroxide, as target molecule.

2.3. *Irradiation conditions.*

Irradiation experiments were performed with a 500 W mercury/xenon lamp (Oriel instruments) equipped with an IR water filter to avoid the overheating of the suspensions. Simulated solar light was obtained by applying a 400nm cut-off filter that let to pass about 5% of UV light in the UV A region.

2.4. *Photo-generation of Reactive Oxygen Species.*

The photo-generation of radical species by TiO_2 powder has been evaluated following a procedure already reported by some of us [6-8]. Briefly, hydroxyl and carboxylate radicals were detected by using DMPO (5,5-dimethyl-1-pyrroline-N-oxide, Alexis Biochemicals, San Diego, CA) and superoxide anion radicals by using PBN (N-tert-butyl-α-phenylnitrone, Sigma-Aldrich, St.Louis, MO) as spin trapping agents. The generation of singlet oxygen was monitored by employing 4-oxo-TMP (2,2,6,6-tetramethyl-4-piperidone, Sigma-Aldrich) as spin probing agent. All tests were performed with an amount of dust corresponding to a surface area of 1.4 m^2.

2.5. *Scavenging activity*.

Scavenging activity has been evaluated following the procedure already reported [9,10]. Briefly, hydroxyl radicals were generated by Fenton reaction or by irradiating with a UV lamp (ThermoOriel UV lamp) a solution of hydrogen peroxide directly in the EPR spectrometer cavity. The reaction was repeated in the presence of carbon soot. All the experiments were repeated at least twice.

2.6. *Oxidative damage to plasmid DNA.*

The reactivity of powders toward double stranded supercoiled plasmid pYES2 (Invitrogen) were been investigated in order to evaluate the potential of samples to cause direct oxidative damage [11]. All experiments were performed with ~ 0.2 mg of powder suspended in 30 µl of milliQ water and then vortexed. To this suspensions 3 µL of DNA solution (concentration 100-150ng/µL) were added and then exposed to a simulated solar light (UV-Vis lamp using a filter having a cut-off of 400nm) for 20 minutes. As a control, DNA was also irradiated in the absence of any powders in order to exclude a direct damage to this molecule. After irradiation time the suspension was centrifuged and the supernatant used for agarose gel electrophoresis analysis. The samples were loaded on a 1% agarose gel (Promega) and, after electrophoresis; DNA bands were stained and visualized with ethidium bromide (Promega).

2.7. Oxidative damage to linoleic acid

The reactivity of powders toward linoleic acid has been investigated in order to evaluate the potential of sample to cause oxidative damage directly to lipids following a procedure previously reported [7]. Briefly, powders and linoleic acid were continuously stirred under the ambient light at 37 ºC for 72 h and then the formation of MDA was evaluated. The assay is based on the reactivity of MDA, a colorless end product of degradation, with tiobarbituric acid (TBA) to produce a pink adduct that absorbs at 535 nm.

2.8. Oxidative damage to proteins

Bovine serum albumin (BSA) (Sigma-Aldrich, Germany) has been chosen as model protein. All experiments were performed with ~ 5 mg of powder suspended in 50 µl of phosphate buffer 5 mM pH 7.4 and then sonicated for 5 minutes. To this suspensions 50 µL of BSA solution (1 mg/ml in phosphate buffer 5 mM pH 7.4) were added and then exposed to a simulated solar light (UV-Vis lamp using a filter having a cut-off of 400nm) for 1 hour. After irradiation time 10 µL of SDS 10% were added in order to eliminate adsorbed proteins at the powder surface and then centrifuged (10 minutes at 10000 rpm). The supernatant, boiled at 100°C in the presence of LAEMMLI solution, was used for SDS-PAGE electrophoresis analysis.

3. Results and discussion

Particles may generate reactive oxygen species by a direct mechanism (surface-derived ROS), or by an indirect one relying on the alteration of mitochondrial functions or the activation of cells of the immune systems (cell-derived ROS) [12-14]. Oxidative stress may also derive by the release of redox-active ions from particles to biofluids [15] or following the depletion of endogenous antioxidants by adsorption or reaction with the particle surface [16,17]. Finally, damage may follow the direct reaction of biomolecules with particles.

Both generation of ROS and direct damage to biomolecules are related to the existence of surface sites accessible to the fluid and able to undergo redox cycling. The chemical nature of these reactive sites depends upon the type of solid. One of the most important reactions occurring at the surface of an inorganic material leading to the generation of ROS is the Fenton reaction, i.e. the generation of hydroxyl radicals through the reaction of hydrogen peroxide with metal ions at a low oxidative state:

$$M^{(n)} + H_2O_2 \longrightarrow M^{(n-1)} + HO^{\bullet} + HO^{-}$$

This reaction was reported to occur in the case of iron containing minerals [18] or particles deriving by grinding covalent solid e.g. quartz [12].

3.1. Fenton-like reactivity

The ability to generate hydroxyl radicals in the presence of hydrogen peroxide has been evaluated by using EPR spectroscopy/spin trapping technique. Figure 1 reports the EPR spectra obtained by

incubating titanium dioxide, amorphous silica or carbon nanoparticles in the presence of the spin trap DMPO and hydrogen peroxide (Figure 1). $FeSO_4$ was used as positive control: in this case the typical four lines EPR spectra of DMPO/ $HO^{\bullet}$ was observed.

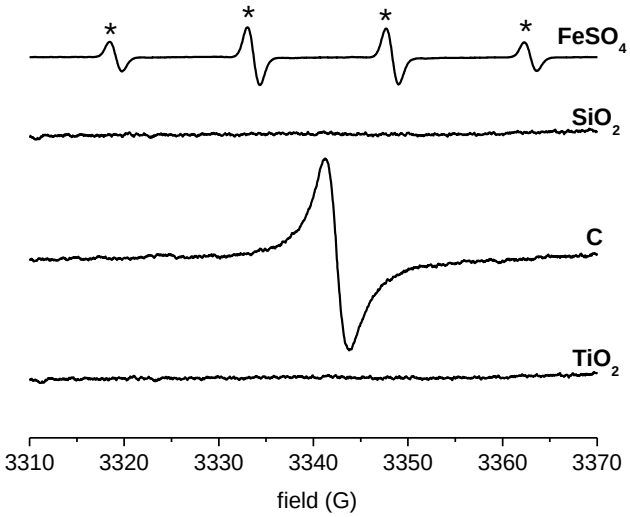

Figure 1: Generation of hydroxyl radical from hydrogen peroxide in the presence of $FeSO_4$, SiO_2, carbon particle (C) and TiO_2. * corresponds to the typical signal of DMPO-HO adduct.

Conversely, no signal was observed in the presence of the powders suggesting that all these materials are unable to reduce hydrogen peroxide. Note that the isometric sharp signal at field 3330-3340 G observed for carbon soot is due to intrinsic carbon centred free radicals, in the bulk of the material [19].

The reaction was performed in the dark since TiO_2 reacts with hydrogen peroxide if activated by UV light generating hydroxyl and superoxide radicals thorough a different mechanisms [7]. Titanium dioxide is in fact known to be a potent photo-catalyst. It generates high amount of reactive oxygen species (ROS) also in the absence of hydrogen peroxide when exposed to UV light in wet conditions. Under UV irradiation charge separation occurs in the bulk of the oxide leading to the promotion of an electron in the conduction band and to the formation of a hole in the valence band. When the charge carriers reach the surface of the solid reduction and oxidation reactions may occur following the interaction with the surrounding medium. The redox potential of charge carriers allow forming highly reactive radical species such as superoxide anion radicals ($O_2^{\bullet -}$), through electron transfer to O_2, and hydroxyl ($HO^{\bullet}$) radicals through hole interaction with water and singlet oxygen ($^1O_2$). The photo-generated holes may also oxidize organic molecules generating carbon-centered free radicals. A comprehensive evaluation of the ROS generated by TiO_2 may be obtained by EPR spectroscopy. The EPR spectra obtained by incubating the TiO_2 sample in solutions containing different spin trap or probe molecules under simulated solar light are reported in figure 2 and compared with the spectra obtained in the dark. Three types of reactions have been considered.

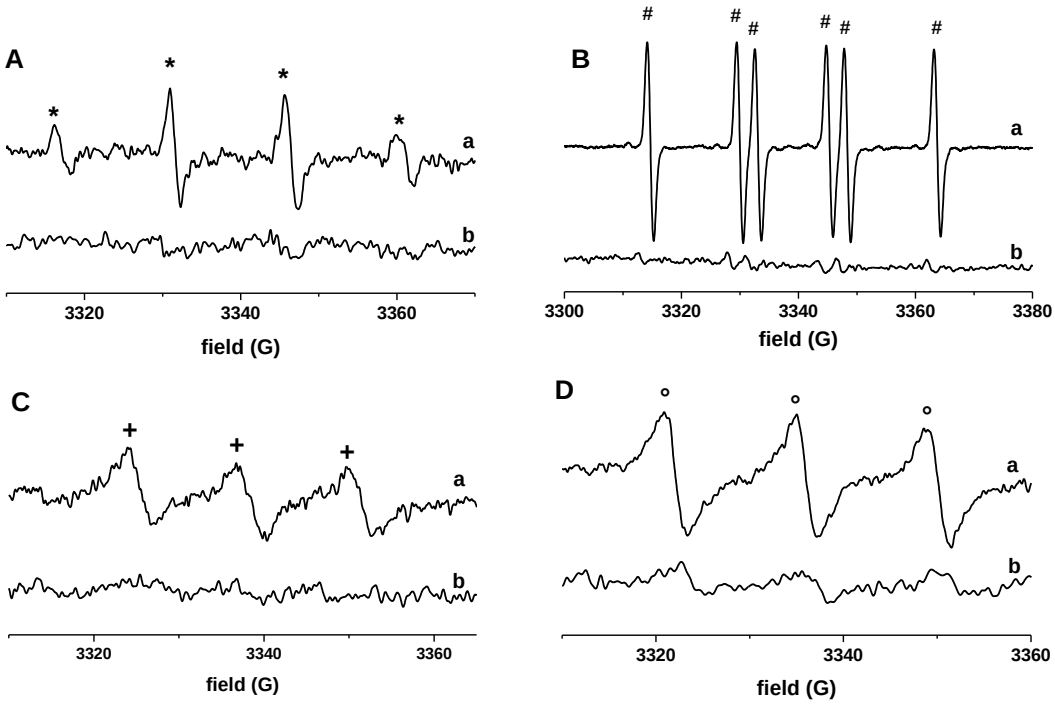

Figure 2: Generation of free radicals by TiO_2 under simulated solar light irradiation (spectra a) or in the dark (spectra b). A) hydroxyl radicals ($HO^{\bullet}$) B) carboxylate radicals ($CO_2^{\bullet-}$) and C) superoxide anion radicals ($O_2^{\bullet-}$) and D) generation of the reactive specie singlet oxygen ($^1O_2$). *, #, + and ° correspond to the typical signal of hydroxyl, carboxylate, superoxide and singlet oxygen signal respectively.

1. The generation of hydroxyl radicals, among ROS the most aggressive species, through oxidation of water by photo-generated holes (h^+):

$$h^+ + H_2O \rightarrow HO^{\bullet} + H^+$$

In agreement with what was previously reported [6-8,20] under irradiation TiO_2 generates large amount of hydroxyl radicals while no radical generation occurs in the dark (panel A).

2. The generation carbon centered radicals by organic molecules (here sodium formiate as model molecule) reacting with photo-generated holes (panel B):

$$h^+ + HCO_2^- \rightarrow CO_2^{\bullet-} + H^+$$

A remarkably intense six lines EPR spectrum correspondent to the trapped $CO_2^{\bullet-}$ radicals was observed under UV irradiation. Albeit much less intense a signal was observed also in the dark.

3. The reduction of oxygen to superoxide radicals:

$$Ti^{4+} + e^- + O_2 \rightarrow O_2^{\bullet-}$$

The typical signal of $O_2^{\bullet-}$ was observed under UV irradiation (Panel C) while no signal was detected in the dark.

4. The generation of singlet oxygen. Being diamagnetic this species is not detected by EPR. However, it may react with the spin probe 4-oxo-TMP to give a nitroxide radical as previously reported by other authors [8]. TiO_2 generates large amount of singlet oxygen when irradiated by UV light (Panel D). A residual photocatalytic activity was also observed in the dark.
Oppositely to titanium dioxide carbon soot and amorphous silica, albeit irradiated with simulated solar light, did not show any activity in generating free radicals.

3.2. Scavenging of free radicals

Beside several materials exhibit at the surface redox active sites able to induce oxidative stress, there are some other that are intrinsically unable to generate free radicals. Amorphous silica and carbon are among them. However, there are numerous evidences that some carbon-based materials (e.g. fullerenes, carbon nanotubes and carbon black) may act as free radical acceptors [9,21,22]. The susceptibility of carbon-based materials to radical attack is well known and has been exploited to introduce functionalities at their surface [23-25]. Some of us previously reported that MWCNTs was able to scavenge oxygenated free radicals and in particular hydroxyl radicals, the most reactive among ROS [9,10]. This property makes these materials promising in all applications where radical reaction need to be controlled such as stabilizing additive for composite [26,27] and in medicine to prevent free-radicals mediated diseases (tumor, cardiovascular diseases and neurodegenerative disorder) [28].

Carbon soot, used in this study as model for carbon particles, derive from incomplete combustion processes or pyrolysis of carbon-containing materials, such as waste or fuel oils, diesel fuel, coal, wood, paper plastic and rubber. It is mainly composed by elemental carbon partially organized in graphenic/graphitic structures. Like other carbon based nanomaterials it scavenges $HO^{\bullet}$ free radicals. Hydroxyl radicals may be generated by irradiating with a UV lamp a solution of hydrogen peroxide or by using the Fenton reaction (H_2O_2, $FeSO_4$). If the reaction is performed in the presence of a spin trap molecule an intense EPR signal is obtained (Figure 3 spectra a). When the reaction is repeated in the presence of carbon soot the signal disappear (Figure 3 spectra b) confirming the ability of carbon based material to scavenge free radicals.

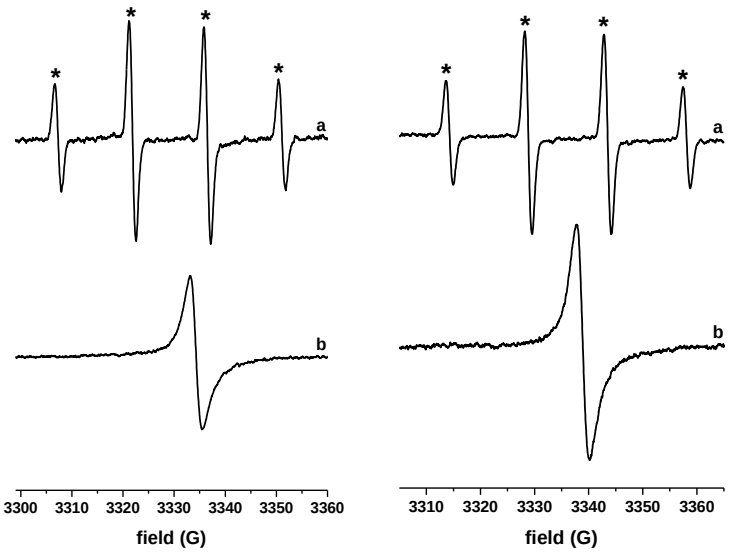

Figure 3: Scavenging activity of soot toward hydroxyl radicals generated by photolysis of H_2O_2 (A) and Fenton reaction (B). a) EPR spectra recorded in the absence of carbon soot; b) EPR spectra recorded in the presence of carbon soot. * corresponds to the typical signal of DMPO-HO adduct.

As reported elsewhere the mechanism of scavenging likely occurs through the addition of radicals to the particles [18]. Amorphous silica did not shown any free radicals scavenging activity in agreement with what previously reported [9].

3.3. Damage to biomolecules

Reactive surface sites also react with organic molecules; the reaction with sodium formiate reported above is an example. Biomolecules may also undergo degradative reactions initiated by surface reactive sites. Therefore the ability to generate ROS is not the only parameter to be considered in the evaluation of the oxidative potential. The capability to directly damage lipids, protein and DNA may be evaluated by cell-free experiments.

Lipids peroxidation was here evaluated by measuring the amount of malonyldialdehyde (MDA) generated after the reaction of linoleic acid with titanium oxide and amorphous silica nanoparticles after 72 h of incubation under ambient light irradiation (Figure 4A). The oxidative potential damage of proteins by titanium oxide and amorphous silica nanoparticles was evaluated under simulated solar light irradiation for 1 h, by using bovine serum albumin (BSA) as model protein (Figure 4B).

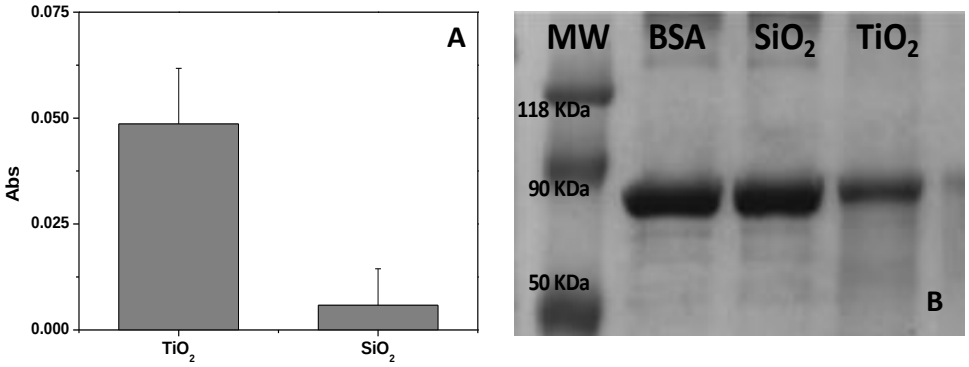

Figure 4: Oxidative degradation of (A) linoleic acid and (B) BSA protein.

As expected under irradiation TiO_2 oxidized both linoleic acid and BSA while amorphous silica did not.

The oxidative potential on DNA by titanium dioxide, amorphous silica and carbon nanoparticles was evaluated under simulated solar light irradiation. Results are reported in figure 5.

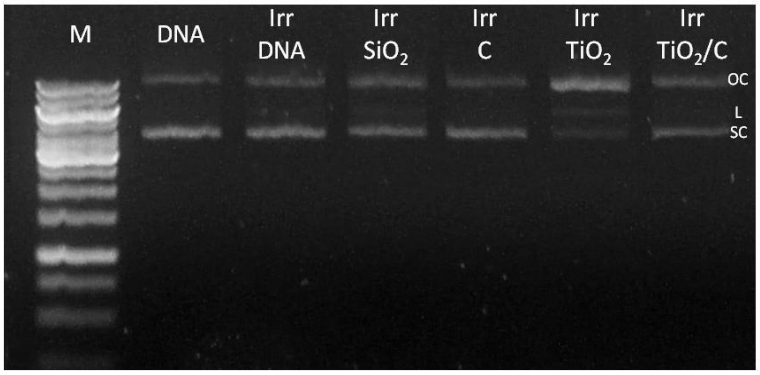

Figure 5: Photo induced damage to double strand supercoiled plasmid DNA. M correspond to DNA weight marker. SC corresponds to supercoiled DNA form, L to linear DNA form and OC to open circular DNA form.

DNA damage (breaks) was performed by irradiating DNA in the presence of powders for 20 minutes and analyzing the supernatant by agarose electrophoresis. Irradiated DNA (Figure 5, column 2) mainly remained in the supercoiled circular (SC) form as well as non-irradiated DNA (column 1) suggesting that direct irradiation did not damage DNA. Moreover, in both cases a band corresponding to open circular form (OC) was present, indicating the presence of DNA already damaged, probably during the plasmid DNA preparation. Addition of both amorphous silica (column 3) and carbon soot (column 4) did not modify the supercoiled/open circular DNA pattern suggesting that no damage occur in the presence of both powders. On the contrary, addition of TiO_2 powder (column 5) caused an increase of open circular form DNA and a partial conversion into linear (L) form of DNA (very low intensity band) suggesting damage to DNA caused by this powder. The contemporary addition of TiO_2 and carbon soot (column 6) partially reversed the damaging effects of TiO_2 powder on DNA. In fact, the bands corresponding to SC and OC forms of DNA show equal intensity indicating that a damage still occurred, but the absence of L form indicates that the damage is lower than that caused by TiO_2 alone suggesting a protective effect of carbon soot. The present data suggest that, oppositely to silica, carbon nanoparticles are not inert but are able to actively interact with the cellular ROS homeostasis by acting as active antioxidants.

4. Conclusions

The growing number of engineered nanoparticles which will potentially enter in the market makes urgent the need of screening protocols able to predict their toxic potential. The availability of validated high throughput screening tests will accelerate both the definition of SARs for nanomaterials and the assessment of the risk related to their exposure. However, when performing screening tests the peculiar chemical properties of each nanomaterial need to be considered. Furthermore, a detailed knowledge of the chemical processes, which may occurs at the nanoparticle/bio-systems interfaces, may help in understanding their behavior in vivo. Integrated chemical screening tests like the one proposed here might be promising tools for the understanding of such processes at a molecular level.

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