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Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay: A quantitative method for oxidative stress assessment of nanoparticle-treated cells

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ABSTRACT

No consensus exists on how to address possible toxicity of nanomaterials as they interfere with most *in vitro* screening tests based on colorimetric and fluorimetric probes such as the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay for detection of oxidative species.

In the present research, nanomaterial interaction with DCFH-DA was studied in relation to its nature and/or assay conditions (cell-based and time exposure) by incubating Rhodamine (Rhd)-labeled 25 nm and 50 nm silica (SiO_2), naked and oleic acid coated magnetite, (Fe_3O_4) and maghemite (Fe_2O_3) iron oxide, titanium dioxide (TiO_2) and poly(ethylene oxide)-poly(lactide/glycolide) acid (PLGA-PEO) nanoparticles (NPs) with metabolically active rat hepatocytes for 4 and 24-h periods. Data indicated that nanoparticle uptake correlated with quenching of dye fluorescence emission. In spite of their masking effect, the oxidative potential of NPs could be detected at a limited threshold concentration when exposed for periods of time longer than those frequently used for this test. However, changes in the experimental conditions did not systematically result in free radical formation for all nanomaterials tested.

Overall data indicate that despite the quenching effect of nanoparticles on DCFH-DA assay, it can be considered as a useful tool for quantitative measurement of NPs-induced oxidative stress by minor modifications of standardized protocols.

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1. Introduction

Nanotechnology is a multidisciplinary field which has achieved important breakthroughs through the integration of bioengineering, molecular biology, diagnostics and therapeutics (Nahar et al., 2006). Despite scientific interest and promising potential in a wide range of applications in our daily life, ranging from burns, wound dressings, sunscreens and cosmetics to fuel cells, tyres, optics, clothing, and electronics, there is still a lack of a consensus approach to address potential toxicity of NPs. Although the raw polymer in bulk form may be inert and biocompatible, nanomaterials often behave differently from their bulk counterparts. Their biological activity and biokinetics are dependent on a series of physicochemical parameters including size, shape, chemistry, charge, and surface modifications among others (Deng et al., 2009; Oberdöster et al., 2009). In addition, a number of recent studies highlight the importance of validation of *in vitro* tests employed for the evaluation of NP toxicity as they may be biased and not accurately reflect their actual toxicity in living organisms. Due to NP reactivity, *in vitro* assay compatibility is a very important requirement, which cannot be overlooked as it might have a dramatic impact on the validity of the results and therefore, on the conclusions regarding safety assessment of nanomaterials (Doak et al., 2009).

There is an increasing concern regarding potential interactions among NPs and colorimetric and fluorescent dyes used in different *in vitro* tests as it has been shown that they might lead to inaccurate absorbance and fluorescence measurements. The extent of such interaction depends on the NP-dye systems being used and is therefore difficult to predict in advance. For example, carbon based nanomaterials have the capacity to absorb dyes onto their surface altering their properties. Therefore, single-walled carbon nanotubes (SWCNTs) appear to interact with some tetrazolium salts such as 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan employed in the MTT colorimetric assay, but they do not interact with others such as WST-1, INT, XTT (Wörle-Knirsch et al., 2006; Davoren et al., 2007). In the same manner, dextran-coated ultrafine superparamagnetic iron oxide nanoparticles (dUSPION) also interfere with the tetrazolium salt-based MTS cell viability colorimetric assay (Doak et al., 2009). Fluorescent dyes are modulated as well

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by NPs. Thus silver (Ag) NPs affect acridine and coumarin dyes leading to spectral changes that result in a fluorescence emission enhancement or quenching at low or high concentrations of Ag NPs, respectively (Sabatini et al., 2007). Recently, Griffiths et al., 2011 demonstrated, using a cell-free system, the relation between the quenching effect of dUSPION on fluorimetric dyes such as calcein, DCFH-DA and 3′-(*p*-aminophenyl) fluorescein (APF) and the amount of NPs, pointing out that control experiments are essential to establish NPs concentration thresholds prior to the use of such probes for the assessment of cell viability and oxidative stress responses following NP exposures. More recently, Kroll et al., 2012 confirmed particle interference with the DCFH-DA test and other cytotoxicity assays (MTT, LDH, IL-8) using a wider range of engineered nanoparticles and the adenocarcinomic human alveolar basal epithelial (A549) cell-based tests. In this study, interference occurred in a highly concentration-, particle- and assay-specific manner thus pointing out that most particle interference could be prevented by altering assay protocols and lowering particle concentration.

In order to investigate the feasibility of this assessment this study aims to evaluate the effect of a set of NPs used mostly for medical purposes on the DCFH-DA fluorescence test for the evaluation of oxidative stress in liver hepatocytes. The assessment of the lack of interference due to NPs is of importance as oxidative stress is one of the most relevant mechanisms of cellular toxicity, and the liver is the major site for biotransformation and defence of orally and intravenously administered foreign materials and xenobiotics. Interference assays involved the measurement of fluorescence due to NPs in cell-free incubation media (i.e. without fluorescent probes), the effect of increasing concentrations of NPs on both cell viability and ROS measurements with time, as well as NP uptake. Moreover, synergistic effects between NP and t-butyl-hydroperoxide (tBHP) were also assessed for Fe_2O_3 and Fe_3O_4 NPs.

2. Material and methods

2.1. Materials

Naked and 3% oleic acid-coated magnetite (Fe_3O_4) and maghemite (Fe_2O_3) NPs were purchased from PlasmaChem GmbH (Berlin, Germany), as a ~5–7% nanosuspension in water. Aqueous solutions of Rhd-labeled SiO_2 NPs of two average particle sizes (25 and 50 nm) were obtained from Corpuscular Inc. (NY, USA). Nanoparticles (PLGA-PEO) consisting of polylactic coglycolic acid (PLGA) copolymer and Poloxamer (Polox) were prepared by Advancell (Santiago, Spain) as a suspension in water. TiO_2-P25 titania powder and 5-(and-6)-carboxy-2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) were purchased from Degussa (Cinncinati, USA) and Invitrogen (Paisley, UK), respectively. Average particle sizes of NPs are summarized in Table 1. Rat collagenase was obtained from Roche (Barcelona, Spain). Ham's F-12, Williams' medium and calf serum were acquired from Gibco (Madrid, Spain).

2.2. Isolation and culture of rat hepatocytes

Hepatocytes were obtained from 200–300 g Sprague Dawley male rats by perfusion of the liver with collagenase as described elsewhere (Gómez-Lechón and Castell, 1998). The cell viability of the suspension was assessed by trypan blue exclusion and resulted in greater than 85% viability. Cells were seeded at a density of 8×10^4 viable cells/cm^2 in Ham's F-12/Williams' (1:1) medium supplemented with 170 μg/mL sodium selenite, 2% calf serum, 0.2% bovine serum albumin, 50 mU/mL of penicillin, 50 μg/mL of streptomycin and 10 nM insulin. Cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere. Unattached cells were removed by changing the medium 1 h after cell seeding. Experimental procedures were applied 24-h after plating.

Table 1
List of tested NPs.

Investigated NPs	Commercial name/ supplier	Average size of primary particles (nm)
SiO_2 25 nm (Rhd-labeled)	*Corpuscular Inc*	26
SiO_2 50 nm (Rhd-labeled)	*Corpuscular Inc*	52
TiO_2	*P25/Degussa Evonik*	22
PLGA	*Advancell*	120
Fe_2O_3	*PlasmaChem*	6
Fe_3O_4	*PlasmaChem*	8
Fe_3O_4 (oleic acid coated)	*PlasmaChem*	8

2.3. Nanoparticle dispersion protocols

SiO_2, PLGA-PEO and iron oxide NPs were provided as suspensions in water. No further dispersion was needed. Tubes were vortex-shaken for a few minutes just before use and thereafter diluted in water to obtain 2 mg/ml stock solutions. Following this, serial dilutions of stock solutions in the 0.384–240 μg/ml range were made in cell culture media to obtain the full exposure range.

Stock solutions of TiO_2 NPs were made by suspending 20 mg of NPs in 10 mL of serum-free medium containing 15 mM Hepes buffer. The suspension was sonicated for a total of 3 min (nine times for 20 s to allow the cooling down of the solution by cold water) at 60 W with the help of an induced ultrasonic probe (Branson, UK). Again, serial dilutions in culture medium were made to obtain NP solutions with concentrations ranging between 0.384–240 μg/ml.

2.4. ROS assay

Rat hepatocytes seeded on 96 well/plates were incubated with the carboxy-2',7'-dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe for 40 min. At the end of this period, medium was removed and cells exposed to the NPs under investigation at a concentration range between 0.384–240 μg/ml. After incubating exposed cells at 37 °C for 4 and 24 h, fluorescence was measured for each time period at 485 nm (excitation) and 527 nm (emission) wavelengths on a microplate reader (Molecular Devices Spectra MAX Gemini X). For those incubations set up to investigate a putative synergism of iron oxide NPs with tBHP, cells were concomitantly incubated for 4-h with both reagents at the indicated concentrations. Fluorescence emission was measured as described earlier.

All assays were performed in at least four individual experiments, each comprising no less than six replicates.

2.5. Quantification of cell viability

Increasing concentrations of NPs in the 0.384–240 μg/ml interval were added to cultures after medium renewal. After a 24-h incubation, microtiter plates were washed twice with 50 μl of PBS/well, and cytotoxicity assessed using the 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) test (Carmichael et al., 1987).

Three independent experiments of six replicates each were performed to assess NP toxicity.

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