

In vitro hazard assessment of nanoparticles developed for biomedical applications

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In vitro hazard assessment of nanoparticles developed for biomedical applications







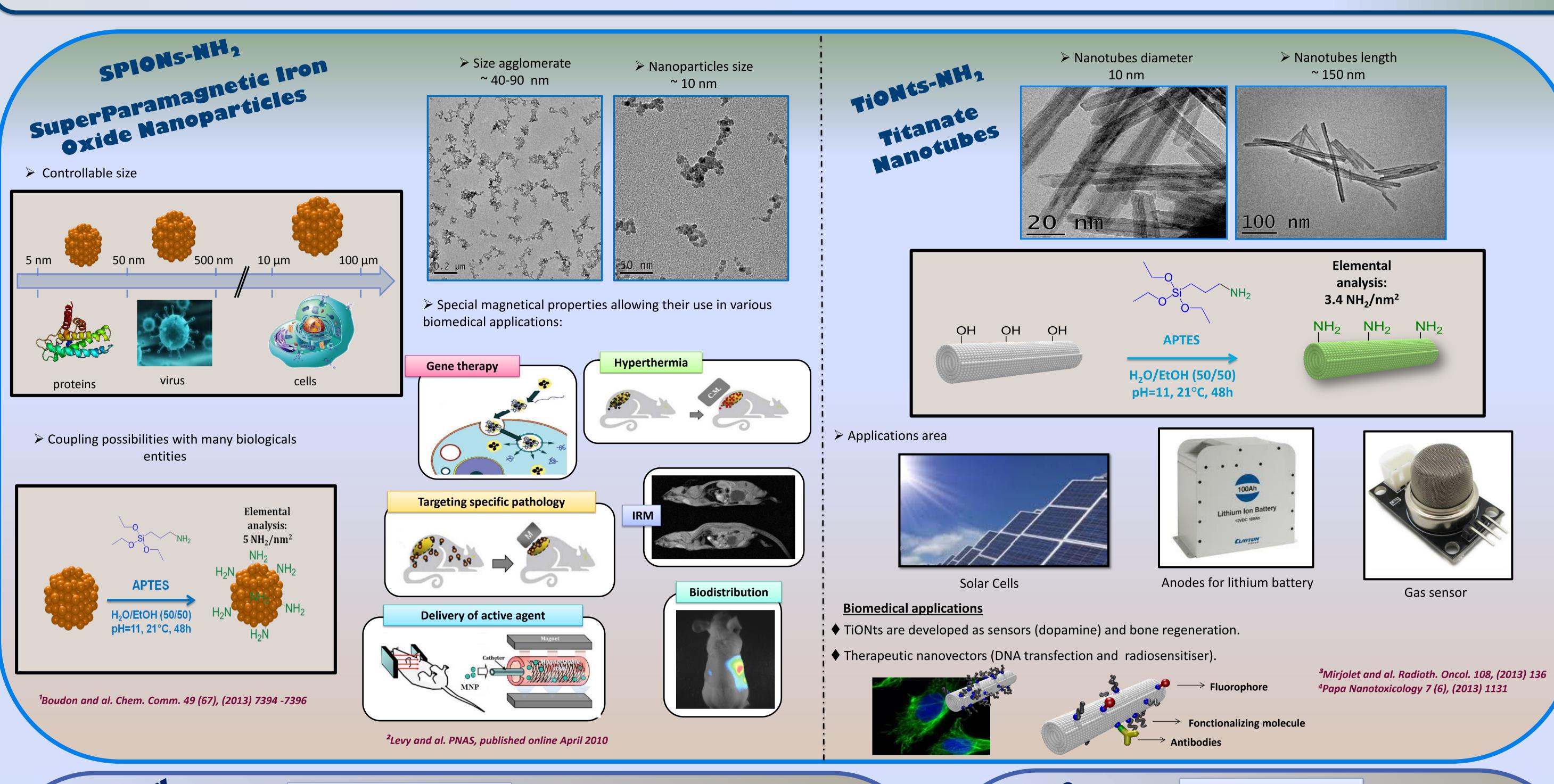
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Nanotechnology is a growing sector in industry for twenty years and nanomaterials are currently used in numerous industrials applications. Their interest is based on their dimensions and their shape that give them very particular technological and biological properties. However, the development of these nanoparticles, their industrial preparation and integration into various products already involve an initial increased of human exposure (via inhalation, percutaneous and oral route). Another exposure pathway exists and is represented by drug injections. The risks associated with these new technologies and new products are still unclear. After a characterization step (zeta potential, TEM, TGA, DLS, XPS, IR measurements ...), different toxicological endpoints were analyzed in vitro. However, bioassay protocols need to be adapted to nanoparticles. Cytotoxicity was assessed with the RNA synthesis inhibition assay, which is an early very sensitive sublethal bioassay. Oxidative stress was detected using DCF-DA assay for a sensitive and rapid quantitation of oxygen-reactive species. For genotoxicity, the comet assay performed in presence of Formamido-Pyrimidine-Glycosylase (FPG) detects low levels of DNA damage relative to DNA oxidative damage. Finally a short-term cell transformation assay (CTA) was performed, using the Bhas 42 cell line to distinguish between tumor initiators and promotors properties. Nanoparticles (SPIONs and titanate nanotubes (TiONts)) didn't induce any cytotoxic, genotoxic and oxidative response after a 24h treatment with concentrations up to 100 μg/mL in the human HepG2 cell line. However, TiONts induced a positive dose dependent response in the CTA assay at the initiation step (1-10 μg/mL).



Comet assay- fpg

Very sensitive assay to detect and quantify DNA breaks. The presence of formamidopyrimidine DNA glycosylase (fpg) allows the detection of specific genotoxic damages.

Principle: Detect single and double DNA strand breaks and alkali-labile sites after an electrophoresis in alkaline condition (pH = 13) and DNA staining with a fluorescent intercalating agent (propidium iodide)⁵.

T+ \rightarrow methyl methanesulfonate C₂H₆O₃S (25 μ M)

> No genotoxic effect on a wide range of non-cytotoxic concentrations using the comet assay

on HepG2 cells.

Positive foci

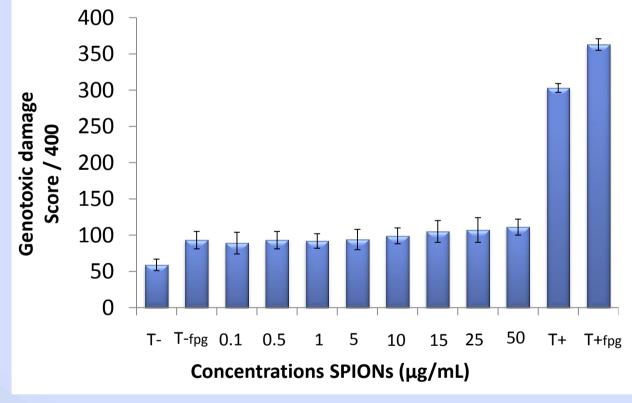


Figure 1: Evaluation of genotoxicity in HepG2 cells incubated 24 hours in the presence of different concentrations of SPIONs. 3 independent experiments were performed and the results were averaged and expressed as score⁶.

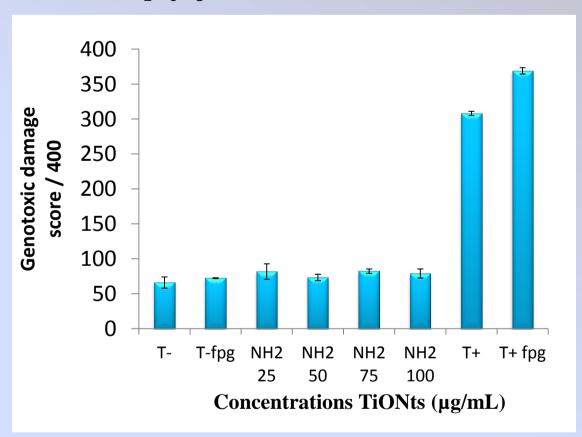


Figure 2: Evaluation of genotoxicity in HepG2 cells incubated 24 hours in the presence of different concentrations of TiONts. 3 independent experiments were performed and the results were averaged and expressed as score⁶.

⁶Collins and al. Molec. Biotech. 26, (2004) 249-261

15 μg/mL

Split DNA

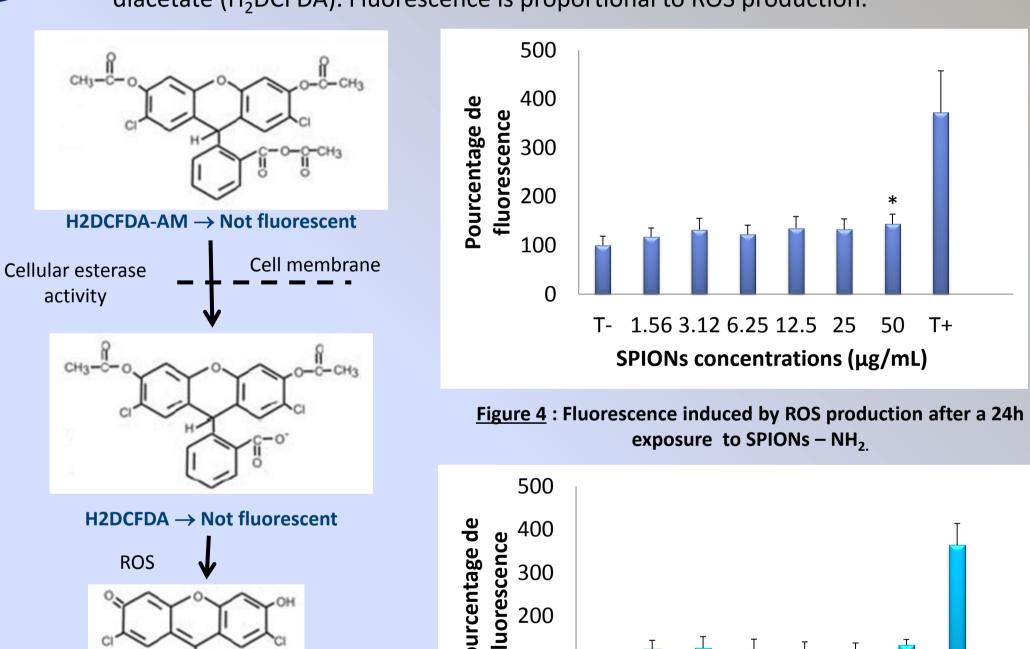
1 μg/mL

Distorted DNA

- (comets class 0 x 0)
- + (comets class 1 x 1) + (comets class 2 x 2)
- + (comets class 3 x 3)
- + (comets class 4 x 4)

DCF-DA assay

Simple assay to detect ROS production by <u>HepG2</u> cell metabolism. Principle: Fluorometric assay using the cell permeant 1',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA). Fluorescence is proportional to ROS production.



T- 1.56 3.12 6.25 12.5 25 50 T+ TiONts concentrations (μg/mL)

Figure 5: Fluorescence induced by ROS production after a 24h exposure to TiONts – NH₂

>ROS production induced by SPIONs and TiONts at high concentrations.

Cell Transformation Assay

Short term assay to determine the carcinogenicity of nanoparticles (90% of carcinogenic substances detected).

Principle: Observations of morphological change of Bhas 42 cells in foci. A significant increase in the number of foci shows a potential carcinogenicity (initiator and/or promotor effect)8.

⁵Domijan and al. Toxicology 222, (2006) 53-59

Negative foci

⁷ EFSA Journal (2011) ⁸ Ayako and al. Mutation Research 725, (2011) 57 - 77

4 x 103 cells/well Chemical treatment Growth phase Promotion assay 14 x 103 cells/well Chemical treatment Figure 6: Initiation and promotion protocols in the Bhas 42 cell transformation assay.

Formula score =

20 TiONts TiONts T+ MCA DMSO $1\mu g/mL 5 \mu g/mL$ 10

μg/mL

Figure 7: Number of foci /well induced after a 3 day exposure with TiONts in the growth phase in the initiation assay.

- DMSO 1μg/mL μg/mL
- promotion assay.
- **2**5 20 **1**5 SPIONS SPIONS T+ TPA DMSO 1µg/mL 5 μg/mL μg/mL

Figure 8: Number of foci /well induced after an 11 day Figure 9: Number of foci /well induced after an 11 day exposure with TiONts during stationary phase in the exposure with SPIONs during the stationary phase in the promotion assay.

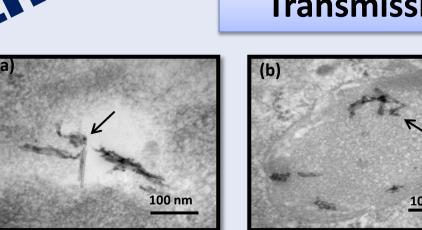
T+ \rightarrow 3-Methylcholanthrene (MCA) 1 µg/mL

 $T+ \rightarrow 12$ -O-tetradecanoylphorbol-13-acetate (TPA) 50 ng/mL

>TiONts induced a statistically significant initiation with a dose-effect (starting at 1 μg/mL) in the Bhas42 cells, promotion response was equivocal with absence of dose response.

SPIONs induced also an equivocal promotion effect without dose response.

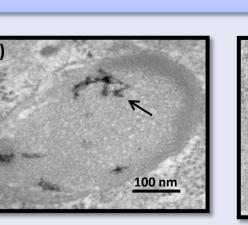
Transmission Electron Microscopy

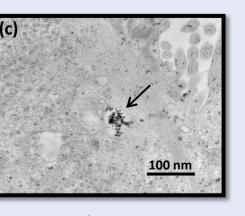


DCF → Fluorescent

T+ → Tert-butyle hydroperoxide (tBHP)

15 μM





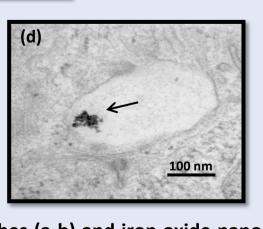


Figure 5: TEM images of HepG2 cells incubated 4 hours in the presence of titanate nanotubes (a-b) and iron oxide nanoparticles (c-d) to a concentration of 100 μg/mL. Figures above show that the TiONts and the SPIONs have a vesicular localization.

A vesicular localization of TiONTs and SPIONSs was observed, probably due to two pathways: endocytosis and diffusion⁹.

⁹ Mirjolet and al. Radiotherapy and Oncology 108, (2013) 136-142

Discussion/conclusions

- **SPIONs** or TiONts were not genotoxic for HepG2 cells.
- \clubsuit TiONts and SPIONs induced ROS production but only at the IC₅₀ (50 µg/mL) determined previously by RNA synthesis inhibition assay. This concentration is however higher than the dose injected by IV to mouse in in vivo assay (30 µg/mL) to check the target tissue and the elimination of nanoparticles.
- TiONts induced initiation and then a potential carcinogenicity, mutagenic assay is running in the laboratory using mammalian cells.
- **Concerning the equivocal promotion data observed with nanoparticles;** experiments are currently performed to check this response.