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In vitro hazard assessment of nanoparticles developed for biomedical applications

A. Luce[ab], Y. Saibi[ab], I. Séverin[b], J. Boudon[a], N. Millot[a], M. C. Chagnon[b]

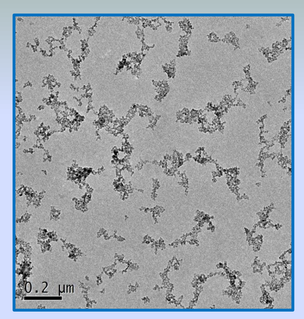
(a) Laboratoire Interdisciplinaire Carnot de Bourgogne (ICB), UMR 6303 - CNRS/Université de Bourgogne
(b) INSERM U866, Derttech « Packtox », Agrosupdijon.

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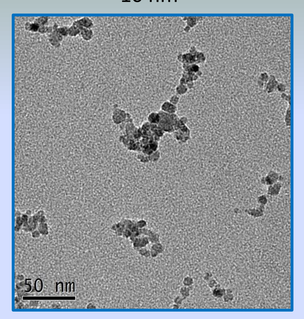
Nanotechnology is a growing sector in industry for twenty years and nanomaterials are currently used in numerous industrials applications. Their interest is based on their dimensions and their shape that give them very particular technological and biological properties. However, the development of these 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).

SPIONs-NH_2 — SuperParamagnetic Iron Oxide Nanoparticles

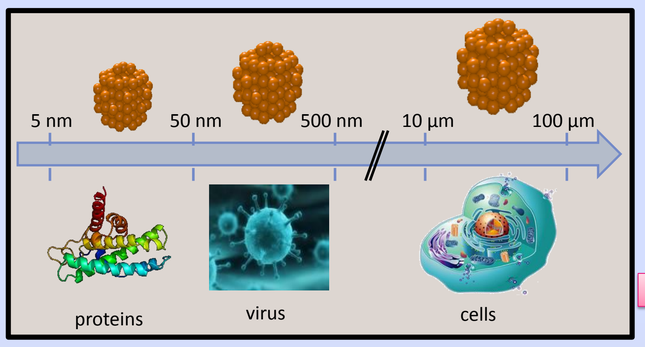
➢ Size agglomerate ~ 40-90 nm



➢ Nanoparticles size ~ 10 nm

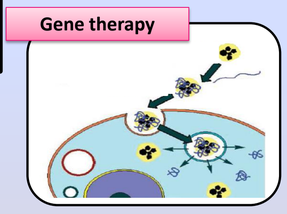


➢ Controllable size

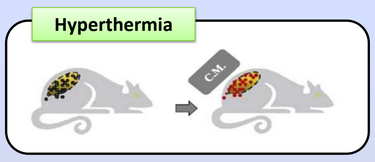


➢ Special magnetical properties allowing their use in various biomedical applications:

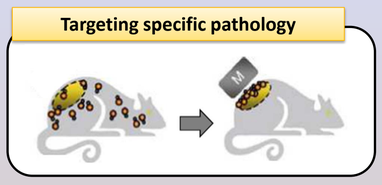
Gene therapy



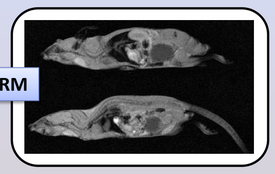
Hyperthermia



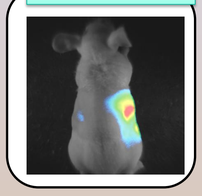
Targeting specific pathology



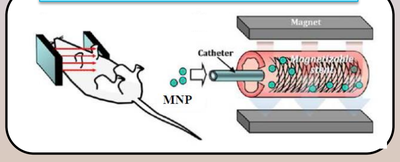
IRM



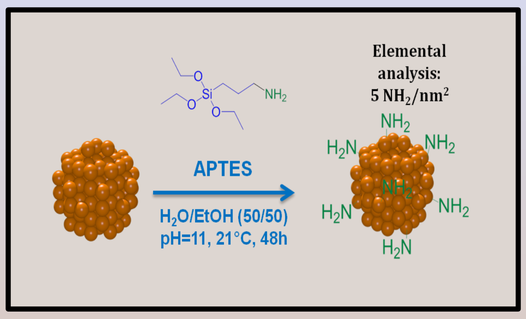
Biodistribution



Delivery of active agent



➢ Coupling possibilities with many biologicals entities

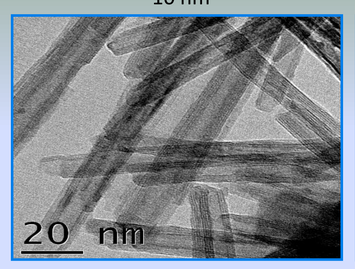


[1]*Boudon and al. Chem. Comm. 49 (67), (2013) 7394 -7396*

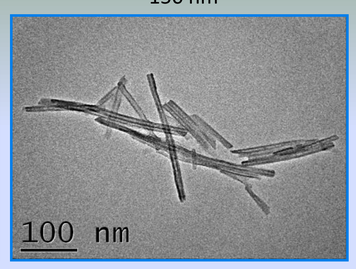
[2]*Levy and al. PNAS, published online April 2010*

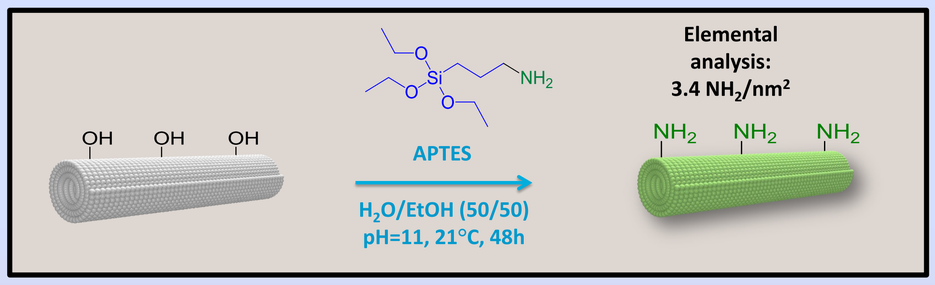
TiONts-NH_2 — Titanate Nanotubes

➢ Nanotubes diameter 10 nm



➢ Nanotubes length ~ 150 nm





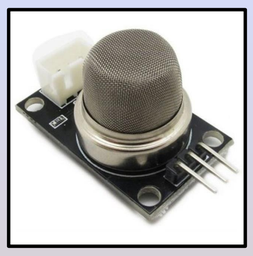
➢ Applications area



Solar Cells



Anodes for lithium battery

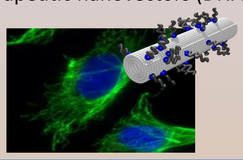


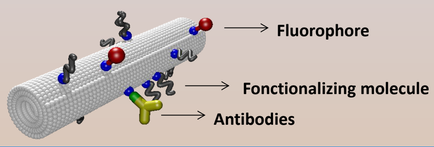
Gas sensor

Biomedical applications

♦ TiONts are developed as sensors (dopamine) and bone regeneration.

♦ Therapeutic nanovectors (DNA transfection and radiosensitiser).





[3]*Mirjolet and al. Radioth. Oncol. 108, (2013) 136*
[4]*Papa Nanotoxicology 7 (6), (2013) 1131*

Genotoxicity assay

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+ → methyl methanesulfonate $C_2H_6O_3S$ (25 µM)

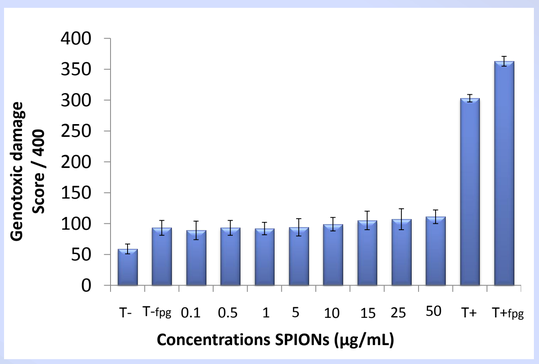


Figure 1 : Evaluation of genotoxicity in HepG2 cells incubated 24 hours in the presence of different concentrations of SPIONs. 3 independent experiments were performed and the results were averaged and expressed as score[6].

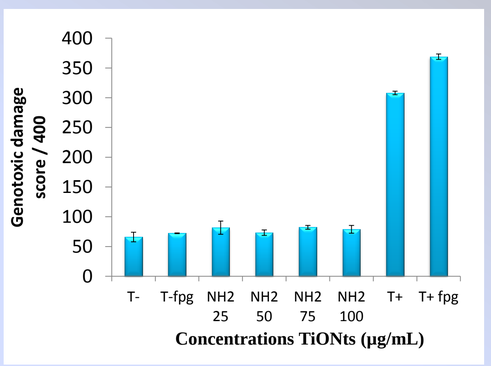
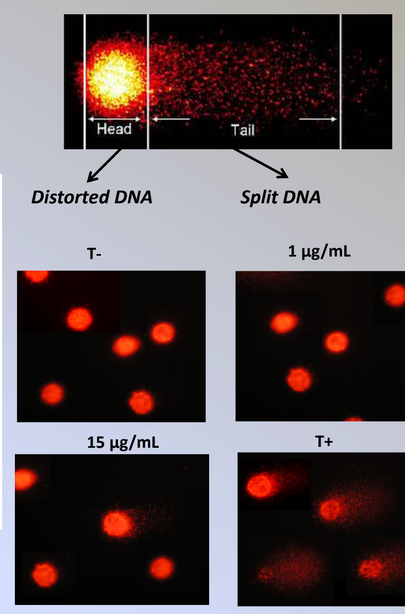


Figure 2 : Evaluation of genotoxicity in HepG2 cells incubated 24 hours in the presence of different concentrations of TiONts. 3 independent experiments were performed and the results were averaged and expressed as score[6].



Formula score = (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)

➢ No genotoxic effect on a wide range of non-cytotoxic concentrations using the comet assay on HepG2 cells.

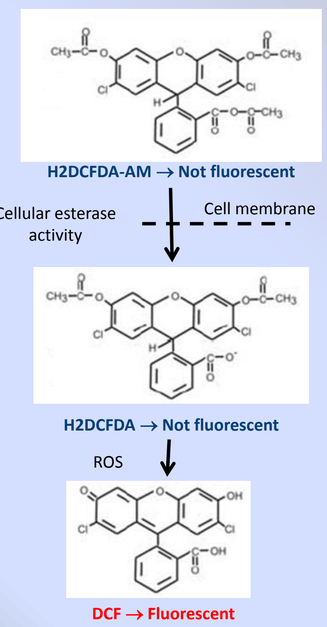
[5]*Domijan and al. Toxicology 222, (2006) 53-59*
[6]*Collins and al. Molec. Biotech. 26, (2004) 249-261*

Oxidative stress

DCF-DA assay

▪ Simple assay to detect ROS production by HepG2 cell metabolism.

Principle: Fluorometric assay using the cell permeant 1',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Fluorescence is proportional to ROS production.



T+ → Tert-butyle hydroperoxide (tBHP) 15 µM

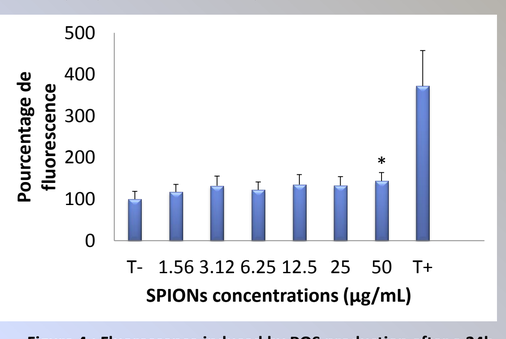


Figure 4 : Fluorescence induced by ROS production after a 24h exposure to SPIONs – NH_2.

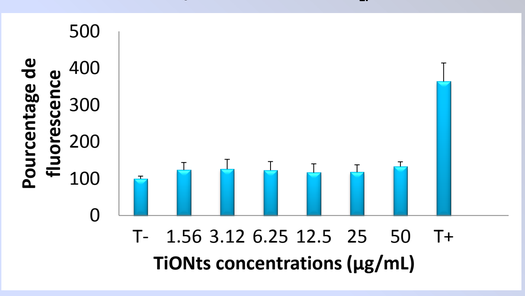


Figure 5 : Fluorescence induced by ROS production after a 24h exposure to TiONts – NH_2.

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

CTA

Cell Transformation Assay

▪ Short term assay to determine the carcinogenicity of nanoparticles (**90% of carcinogenic substances detected[7]**).

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].

Negative foci **Positive foci**

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

Figure 6 : Initiation and promotion protocols in the Bhas 42 cell transformation assay.

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

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

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

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

T+ → 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.

TEM

Transmission Electron Microscopy

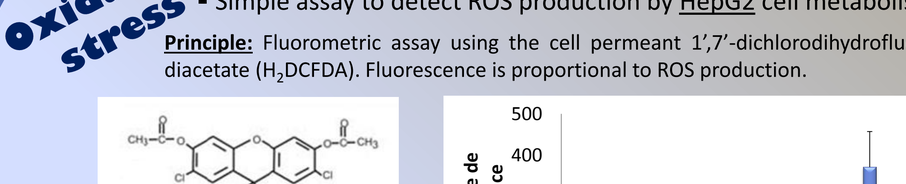


Figure 5 : TEM images of HepG2 cells incubated 4 hours in the presence of titanate nanotubes (a-b) and iron oxide nanoparticles (c-d) to a concentration of 100 µg/mL. Figures above show that the TiONts and the SPIONs have a vesicular localization.

➢ A vesicular localization of TiONTs and SPIONSs was observed, probably due to two pathways: endocytosis and diffusion[9].

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

Discussion/conclusions

❖ **SPIONs or TiONts were not genotoxic for HepG2 cells.**

❖ **TiONts and SPIONs induced ROS production but only at the IC_{50} (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.**