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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 industrial 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 promoters 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<sub>2</sub> SuperParamagnetic Iron Oxide Nanoparticles

➢ Controllable size

➢ Coupling possibilities with many biological entities

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

- Gene therapy
- Hyperthermia
- Targeting specific pathology
- IRM
- Delivery of active agent
- Biodistribution

➢ APTES functionalization: Si-OH + NH2-R >> Si-O-Si-NH2-R  
H<sub>2</sub>O/EtOH (50/50), pH=11, 21°C, 48h  
Elemental analysis: 5 NH<sub>2</sub>/nm<sup>2</sup>

<sup>1</sup>Boudon et al. Chem. Comm. 49 (67), (2013) 7394-7396  
<sup>2</sup>Levy et al. PNAS, published online April 2010

## TiONts-NH<sub>2</sub> Titanate Nanotubes

➢ Nanotubes diameter: 10 nm  
➢ Nanotubes length: ~150 nm

➢ APTES functionalization: Ti-OH + NH2-R >> Ti-O-Si-NH2-R  
H<sub>2</sub>O/EtOH (50/50), pH=11, 21°C, 48h  
Elemental analysis: 3.4 NH<sub>2</sub>/nm<sup>2</sup>

➢ 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).

➢ Fluorophore, Functionalizing molecule, Antibodies

<sup>3</sup>Mirjole et al. Radioth. Oncol. 108, (2013) 136  
<sup>4</sup>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)<sup>5</sup>.

T+ → methyl methanesulfonate C<sub>2</sub>H<sub>6</sub>O<sub>3</sub>S (25 µM)

**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.

<sup>5</sup>Domijan et al. Toxicology 222, (2006) 53-59  
<sup>6</sup>Collins et 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',3'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA). Fluorescence is proportional to ROS production.

H<sub>2</sub>DCFDA-AM → Not fluorescent  
Cellular esterase activity → Cell membrane → H<sub>2</sub>DCFDA → Not fluorescent  
ROS → DCF → Fluorescent

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

➢ 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<sup>7</sup>).

**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 promoter effect)<sup>8</sup>.

Negative foci vs Positive foci

<sup>7</sup>EFSA Journal (2011)  
<sup>8</sup>Ayako et al. Mutation Research 725, (2011) 57-77

**Figure 6:** Initiation and promotion protocols in the Bhas 42 cell transformation 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 SPIONs was observed, probably due to two pathways: endocytosis and diffusion<sup>9</sup>.

<sup>9</sup>Mirjole et 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<sub>50</sub> (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.