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In vitro hazard assessment of nanoparticles developed for biomedical applications

Annette Luce, Y Saibi, Isabelle Séverin, Julien Boudon, Nadine Millot,
Marie-Christine Chagnon

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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-NH₂ 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
H₂O/EtOH (50/50), pH=11, 21°C, 48h
Elemental analysis: 5 NH₂/nm²

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

TiONts-NH₂ Titanate Nanotubes

➢ Nanotubes diameter 10 nm
➢ Nanotubes length ~ 150 nm

➢ APTES functionalization: Ti-OH + NH2-R >> Ti-O-Si-NH2-R
H₂O/EtOH (50/50), pH=11, 21°C, 48h
Elemental analysis: 3.4 NH₂/nm²

➢ 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

³Mirjole et al. Radioth. Oncol. 108, (2013) 136
⁴Papa 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 C₂H₆O₃S (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)

⁵Domijan et al. Toxicology 222, (2006) 53-59
⁶Collins 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 (H₂DCFDA). Fluorescence is proportional to ROS production.

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 detected⁷).

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)⁸.

Negative foci vs **Positive foci**

⁷EFSA Journal (2011)
⁸Ayako 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 diffusion⁹.

⁹Mirjole 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 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.