High throughput comet assay to study genotoxicity of nanomaterials

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Toxicology vs Nanotoxicology

“The dose makes the poison”
Paracelsus
(1493 - 1541)
Nanoparticles (5-50 nm)

Aggregates
Chemically bonded (30-200 nm)

Shear forces

Agglomerates
Van der Waals forces (1-100 μm)

Smallest structure possibly present

Shear forces
Nanoparticle (NP) exposure and mechanism of action on cell

NP exposure

Inhalation
Oral
Intravenous
Dermal

NP uptake
Endocytosis
Activation of receptor

NP releases transition metals
NP surface causes oxidative stress

Increased oxidative stress
Signaling pathways

Nucleus

Genotoxicity
Inflammatory mediators

Inflammation

Primary genotoxicity

Secondary genotoxicity
High-throughput comet assay  HTP-CA

The comet assay (with lesion-specific enzymes) - principle

Cells embedded in agar on microscope slide

Lysis: Triton X-100, 2.5 M NaCl

Nucleoid; supercoiled DNA

± Digestion with lesion-specific endonuclease (FPG)

Alkaline incubation: 0.3 M NaOH, 10 mM EDTA

Electrophoresis: 0.8 V/cm, 30 min

Neutralisation, DAPI stain, fluorescence microscopy

The frequency of damaged bases is given by the increase in DNA breaks in the presence of the specific endonuclease

This assay allows us to estimate the level of strand breaks or other lesions such as oxidised bases

<table>
<thead>
<tr>
<th>Localisation of the gel on slides</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 gels slides format</td>
</tr>
<tr>
<td>5-15μl/drop</td>
</tr>
</tbody>
</table>

Agarose volume
HTP-CA example strategy for testing NPs

**Preparation of NP1 STOCK**

**Characterisation.**
- Nanosight/DLS...

**Uptake..TEM; SEM ...**

NP1...NP2...NP3...NP4

NPs dilutions

Exposure 3 + 24 hr (CA+Cytotox)

Cells + LMA 0.8%

- Exposition
- Fixation
- Dehydration
- Embedding in pure Epon
- ~80 nm sections were cut using a diamond knife and mounted on copper grids
- Transmission electron microscope (120Kv)

12 gel format on slides/48-96 format on GBs

**Cytotoxicity eg. AlamarBlue Others endpoints**
HTP-CA example strategy for testing NPs

In one experiment, for one cell line: 1 NPs – 8 concentration, 2 times of exposure (3 – 24hr). 2 replicates per concentration. 2 duplicates per replicate. 3 treatment (lysis, buffer, FPG) = Total of samples

96 gels/NP/cell line = 8 slides 12 gel format vs 48 slides 2 gel format

384 gels / 4 NPs/cell line

768 gels/4 NPs/ 2 cell lines

SCORING ???
It has been suggested that NPs remaining with cells after embedding might interfere with FPG in the comet assay.

HeLa cells were treated with silica NPs (0, 30, 75 µg/cm²) for 3h and then treated with or without Ro 19-8022 (photosensitiser) + light to induce 8-oxoGua.

No indication that NPs reduce the effectiveness of FPG

Interference of photocatalytically active NPs with the Comet assay?

BEAS-2B cells to particles (TiO2 rutile, TiO2 anatase and CuO, 20 mg/mL) for 3 hr

D. Dark
L1. under lab light after lysis for 111 min
L2. similar to L1 for 313 min.

*Karlsson et al 2014. Environmental and Molecular Mutagenesis*
Six laboratories from the NanoTEST consortium performed the comet assay following the same experimental design and procedures. PLGA-PEO, TiO$_2$, U-Fe$_3$O$_4$, OC-Fe$_3$O$_4$, Fl-25 SiO$_2$, and Fl-50 SiO$_2$ NPs from single batches were tested for genotoxicity.

All *in vitro* studies were harmonized; identical dispersion protocols, exposure time, concentration range, culture conditions, and time-courses were used.

Statistical regression modelling was carried out using the Genstat statistical software package to look at the dose-response association between dose and SBs or dose and net FPG-sensitive sites and determining whether it varied between nanomaterials and between cell lines.
**Blood**: human blood cells—leucocytes, granulocytes, monocytes, etc. TK6

**Kidney**: monkey kidney Cos-1 cells, human HEC

**Central nervous system**: HCEC, EC219, Murine N11 microglial cells

**Lung**: bronchial epithelial cell lines (16HBE, NCIH and Calu-3, and human alveolar type 2 cells A549, HBEC)

**Liver**: hepatocytes, kupffer cells and liver sinusoidal endothelial cells (LSEC), HepG2

**Vascular**: HCEC, EC219, ECp23, HL1

**Digestive system**: Colon HT29, Caco 2, CacoGoblet, CacoReady TM,

**Placenta**: Placenta perfusion, BeWo
## NP characterisation. Primary properties

<table>
<thead>
<tr>
<th></th>
<th>TiO$_2$ 21nm</th>
<th>PLGA 140nm</th>
<th>OC-Fe3O4, coating 8±3nm</th>
<th>U-Fe3O4, no coating 8±3nm</th>
<th>Fluorescent SiO$_2$, 25 nm</th>
<th>Fluorescent SiO$_2$, 50 nm</th>
<th>Nano SiO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal structure</td>
<td>Anatase/rutile</td>
<td>Unknown</td>
<td>Spinel (octahedral)</td>
<td>Spinel (octahedral)</td>
<td>Amorphous</td>
<td>Amorphous</td>
<td>Amorphous</td>
</tr>
<tr>
<td>Chem compos</td>
<td>Ti, O</td>
<td>C, H, O</td>
<td>Fe, O</td>
<td>Fe, O</td>
<td>Si, O</td>
<td>Si, O</td>
<td>Si, O</td>
</tr>
<tr>
<td>TEM/EDX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle concen (%) 1</td>
<td>Not applicable</td>
<td>0.33</td>
<td>26</td>
<td>2.8</td>
<td>-</td>
<td>-</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Shape-TEM</td>
<td>Irregular</td>
<td>Not applicable</td>
<td>Oblong</td>
<td>Oblong</td>
<td>Round/oblong</td>
<td>Round/oblong</td>
<td>Irregular, rectangular</td>
</tr>
<tr>
<td>Crystallite size</td>
<td>15-60</td>
<td>Not applicable</td>
<td>5-12</td>
<td>5-13</td>
<td>15-30</td>
<td>25-50</td>
<td>5-30</td>
</tr>
<tr>
<td>distribution-TEM (nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface area-BET (m$^2$/g)</td>
<td>61</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>226</td>
</tr>
<tr>
<td>Pore volume-BET (mL/g)</td>
<td>0.13</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>0.7</td>
</tr>
<tr>
<td>Surface chemistry</td>
<td>Uncoated</td>
<td>Uncoated</td>
<td>Oleate micelle coating</td>
<td>Uncoated</td>
<td>Uncoated</td>
<td>Uncoated</td>
<td>Uncoated</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>-30.2</td>
<td>-43.4</td>
<td>-31.9</td>
<td>-2.8</td>
<td>-20</td>
<td>-22</td>
<td></td>
</tr>
<tr>
<td>milliQ pH7 (mV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Free oleate (960 ppm), Na (26.000 ppm), Ca (1.300 ppm), K (730 ppm)
Size distribution and stability of oleic acid coated iron oxide in various culture media (Conc.: 0.25 mg/ml)

<table>
<thead>
<tr>
<th>Medium (Conc.: 0.25 mg/ml)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Size stability with time</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>Very large agglomerates,  &gt; 900</td>
<td>&lt; 10 min</td>
</tr>
<tr>
<td>DMEM +10% FBS</td>
<td>Trimodal distribution,    18, 86 and 237</td>
<td>~ 2 days</td>
</tr>
<tr>
<td>DMEM-HG</td>
<td>Very large agglomerates,  &gt; 2000</td>
<td>&lt; 5 min</td>
</tr>
<tr>
<td>DMEM-HG +10% FBS</td>
<td>Bimodal distribution,     36 and 153</td>
<td>~ 3 days</td>
</tr>
<tr>
<td>RPMI</td>
<td>Trimodal distribution,    18, 73 and 232</td>
<td>~ 2 days</td>
</tr>
<tr>
<td>RPMI +10% FBS</td>
<td>Bimodal distribution,     39 and 165</td>
<td>~ 3 days</td>
</tr>
<tr>
<td>DMEM- F12-HAM</td>
<td>Bimodal distribution,     31 and 132</td>
<td>~ 3 days</td>
</tr>
<tr>
<td>DMEM-F12-HAM +10% FBS</td>
<td>Bimodal distribution,     36 and 153</td>
<td>~ 3 days</td>
</tr>
</tbody>
</table>
Biological effect depends on dispersion.

The state of Agglomeration and aggregation of NPs is important.

DNA damage (comet assay) after 24h exposure of EUE cells to TiO$_2$ NPs; 2 dispersions.

Magdolenova et al, Environ Monit, 2012,
The cell line/nanomaterial effect

DNA damage is associated with NP type, time of exposure, and concentrations. Only TiO2 and coated Fe3O4 showed significant genotoxic effect.

blood (TK6, human lymphocytes); kidney (Cos-1 and HEK293); lung (16HBE); placenta (BeWo); liver (hepatocytes and Kupffer cells); human brain vascular system (HCEC).

<table>
<thead>
<tr>
<th>Nanomaterial</th>
<th>Coefficient (per µg/cm²)</th>
<th>s.e.</th>
<th>Coeff/s.e. (t-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OC-Fe₃O₄</td>
<td>0.0083</td>
<td>0.0012</td>
<td>6.44</td>
</tr>
<tr>
<td>U-Fe₃O₄</td>
<td>0.0020</td>
<td>0.0011</td>
<td>1.94</td>
</tr>
<tr>
<td>PLGA-PEO</td>
<td>-0.0013</td>
<td>0.0011</td>
<td>-1.23</td>
</tr>
<tr>
<td>FI-25 SiO₂</td>
<td>0.0021</td>
<td>0.0011</td>
<td>1.97</td>
</tr>
<tr>
<td>FI-50 SiO₂</td>
<td>0.0016</td>
<td>0.0011</td>
<td>1.45</td>
</tr>
<tr>
<td>TiVedisp</td>
<td>0.0029</td>
<td>0.0012</td>
<td>2.53</td>
</tr>
<tr>
<td>TiUPdisp</td>
<td>0.0053</td>
<td>0.0019</td>
<td>2.85</td>
</tr>
</tbody>
</table>
DNA damage in various cells after 24h exposure to NPs

The oleic acid coated Fe$_3$O$_4$ and TiO$_2$ NPs were positive in all types of cells. The OC-Fe$_3$O$_4$ exposure caused significant genotoxicity in contrast to TiO$_2$ UPdisp. OC-Fe$_3$O$_4$ and TiO$_2$ UPdisp can be used as positive control.

Cowie et al Nanotoxicology. 2015
Impact of nanosilver surface charge and surface coating on level of DNA damage

Huk et al. 2015. Particle and Fibre Toxicology
Final remarks

- From available methods – comet assay seems to be suitable with advantage to be used for HTP. However automation of scoring is inevitable.
- Design for NPs testing must be always accompanied with characterization and uptake.
- Dispersion of NMs and exposure conditions are crucial and should mimic real situation
- Concentrations used for genotoxicity studies should be realistic, i.e. relevant to possible exposures.
- Cytotoxicity should be integral part of the genotoxicity
- Relevant positive and negative control should be always included to NPs testing
- Possible interference of nanomaterial with assay should be always considered
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