Critical factors to be considered when testing nanomaterials for genotoxicity with the comet assay

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Abstract

The comet assay is widely used to test the genotoxicity of engineered nanomaterials (ENMs) but outcomes may vary when results from different laboratories, or even within one laboratory, are compared. We address some basic methodological considerations, such as the importance of carrying out physico-chemical characterisation of the ENMs in test-medium, performing uptake and cytotoxicity tests, and testing several genotoxicity-related endpoints. In this commentary, we discuss the different ways in which concentration of ENMs can be expressed, and stress the need to include appropriate controls and reference standards to monitor variation and avoid interference. Treatment conditions, including cell number, cell culture plate format and volume of treatment medium on the plate are crucial factors that may impact on results and thus should be kept constant within the study.

Introduction

Genotoxic compounds are generally mutagenic and thus potentially carcinogenic. It is particularly important that a strategy for genotoxicity testing should cover different mechanisms and endpoints—namely DNA breaks, altered DNA bases, mutations and chromosomal alterations (1,2).

Compared with the parent bulk chemicals, engineered nanomaterials (ENMs) tend to be more reactive in biological systems, on account of the small size and correspondingly large ratio of surface area to mass, and assessment of the potential genotoxicity of ENMs is particularly demanding; a battery of suitable tests, adapted from established genotoxicity tests, need to be rigorously validated by examining their performance with various ENMs, test media and cell types. We discuss the need for standard procedures and quality controls to minimise variation and to avoid obtaining false positive/negative results.

While we concentrate here on DNA damage, we would emphasise the importance of carrying out parallel studies with other endpoints.

Measuring DNA-damaging effects of ENMs with the comet assay

The comet assay (single cell gel electrophoresis) is a simple, robust, reliable and user-friendly method for measuring DNA damage, which has been widely used in nanogenotoxicology studies (3). Miniaturised versions of the assay—with 12 mini-gels per slide (replacing two in the standard assay) or 96 mini-gels on a GelBond film—allow the analysis of numerous types of ENM and concentrations in a relatively short time (4,5). DNA strand breaks are measured together with base alterations, including 8-oxo-guanine, a potent premutagenic lesion detected by digesting DNA with formamidopyrimidine DNA glycosylase.

Generally, for genotoxicity screening of ENMs, virtually any cell type can be used, allowing the study of target organ specificity and cell type sensitivity (6).

Defining conditions for testing ENMs

Published results on genotoxicity of ENMs show high variability even with the same ENM. Reasons for this variability include the...
diversity of methods for handling ENMs and preparing dispersions, variation in size distribution and dispersion stability, and different exposure conditions (7). The dispersion and stability of ENMs are influenced by components present in the medium, such as serum proteins, and by the physicochemical properties of the ENMs (size, shape, charge, surface coating, etc.). Therefore, it is essential to characterise ENMs in the relevant medium and to apply appropriate treatment conditions.

**How concentrations are expressed is important**

The concentration of ENMs is commonly expressed in mass units per volume [μg/ml], per area [μg/cm²] or per cell [μg/cell]. The relationship between the mass units can vary depending on the type of culture plates, amount of medium and number of cells used. Additionally, concentrations can be expressed as number of ENMs per ml [ENMs/ml], per cm² [ENMs/cm²] or per cell [ENMs/cell] as well as surface area of ENMs per ml [ENM cm²/ml], per cm² [ENM cm²/cm²] or per cell [ENM cm²/cell]. The use of concentration per cell seems to be particularly appropriate for ENM testing.

It is recommended that concentrations be expressed in at least two different units, not only as mass but also as number of ENMs or as surface area, since surface properties and size are among those physicochemical properties of ENMs that may impact on genotoxicity and thus these units might be more informative for the comparative evaluation of toxicity of different ENMs. In the case of Ag ENMs tested by us, the results expressed in mass units [μg/cm²] showed that Ag ENM toxicity is size-dependent, with 50 nm particles being most toxic. However, re-calculation of Ag ENM concentrations from mass units to surface area, and number of ENMs per cm² or per cell (Figure 1) indicated that 200 nm Ag ENMs, the largest particles in this particular case, were the most genotoxic (8).

**Deciding on appropriate concentrations for testing**

Concentrations used for genotoxicity studies should be realistic, i.e. relevant to possible human exposures. ENMs have a tendency to agglomerate, and therefore the concentration of ENMs in tests should not exceed the level at which agglomeration is enhanced.

In the case of the comet assay, recommended concentrations should range from nontoxic to around 80% viability, since DNA breakage can be a secondary effect of cytotoxicity and so the use of cytotoxic concentrations could give false positive results.

Appropriate cytotoxicity tests should be part of the testing strategy (9). The most reliable assays are relative growth activity or proliferative capacity, and colony forming ability, addressing cell death and cytostatic effects as endpoints. However, care should be taken with automated counting, since interference can occur at this stage (10). Cytotoxicity should always be tested with the same cells (and ideally in the same experiment) as used in the genotoxicity test.

**Treatment time and assay conditions**

For ENM genotoxicity studies, generally prolonged (at least 24 h) treatment is recommended to ensure uptake by cells and access to DNA when the nuclear membrane is dissolved. For the comet assay, a short (2–3 h) exposure is usually sufficient to demonstrate a positive response (11) and could be included as well.

No modifications of the comet assay (lysis buffer, unwinding and electrophoresis parameters etc.) are necessary when testing ENMs. At least 50 comets per gel (and parallel gels per sample) should be scored to allow reliable statistical analysis.

**The need for experimental controls**

Positive and negative controls should always be included in experiments for quality control, to demonstrate correct performance of the assay, and to ensure reproducibility. Negative controls should show the background level of DNA damage. In contrast, positive controls should show strongly significant effects: for the comet assay, hydrogen peroxide is used to induce strand breaks, and photosensitiser Ro19-8022 with visible light to induce oxidised purines. Additional controls with the ENM solvent should also be included to avoid false positive results.

A challenge for nanogenotoxicity studies is to devise suitable nano-specific positive controls. There are several initiatives currently focusing on selection of ENMs with appropriate properties for use as nano-specific reference standards, but as yet no clear generally accepted candidate has been found and no consensus has been reached.

Reproducibility is crucial for any testing, but especially for ENMs, as there are many factors that may contribute to variability between and within tests. Thus building a database of historical positive and negative controls is good practice for quality assurance.

**Coating materials, solvents and breakdown products of ENMs can affect the toxicity**

Coating materials or stabilizers can cause genotoxicity and thus should also be tested and included in the experimental set-up as additional reference materials. Note also that nanoparticles are good carriers, and if a stabilizer or coating is toxic, low, normally non-toxic concentrations can cause damage owing to their enhanced internalisation into cells. Additional controls to discriminate between coating/solvent/stabiliser effects and an effect of ENMs are of utmost importance (A. Huk et al., unpublished result).

Metal ions released from ENMs can cause production of reactive oxygen via Fenton-like reactions. Therefore it is important to test whether genotoxic effects are caused by the metal ions rather than by the ENMs themselves (A. Huk et al., unpublished result; E. Izak-Nau et al., unpublished result).

**Interference of ENMs with tests**

Properties of ENMs such as adsorption capacity, optical properties, hydrophobicity, chemical composition, surface charge and surface properties, catalytic activities as well as agglomeration can result in interference with standard toxicity tests (10). Agglomeration of ENMs affects their bioavailability to the cell and thus might lead to false positive/negative results.

In the case of the comet assay occasional observation of particles in the ‘comet head’ raised concerns of possible interference of NPs during the performance of the assay (12, 13). Magdolena et al. (14) tested various ENMs (SiO₂, TiO₂, mixed rutile and anatase, Fe₃O₄ coated and uncoated, polylactic co-glycolic acid) and did not find any interference. However, we recommend testing for possible interference of ENMs in the gel, co-incubating them with both untreated cells and cells exposed to a known genotoxic compound (causing DNA strand breaks as well as oxidised DNA lesions). This would be a sensible precaution to be sure that no overestimation or underestimation of damage is occurring.
**Demonstrating cellular uptake**

In assessing ENM toxicity, including genotoxicity, information on uptake is needed. Even if ENMs are not localised within cells, they might be indirectly genotoxic via oxidative stress or inflammation. However, negative results can be trusted only if uptake of ENMs has been demonstrated.

**Conclusions and final remarks**

The comet assay has many advantages, although some might be seen as limitations. For example, the method has high sensitivity, but is capable of detecting damage only over a fairly narrow range, between about 100 and several thousand breaks per cell.

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**Figure 1.** Relationship between size of Ag ENMs and DNA damage induced in A549 cells. Data are from Huk et al. (8) recalculated in terms of mass (A), surface area (B) or number (C) of ENMs per cell. DNA damage (strand breaks) was measured with the comet assay and is expressed as percentage of DNA in comet tails (means from 3 independent experiments, with standard error means). ENM sizes were 50 nm (squares), 80 nm (triangles) and 200 nm (circles).
Higher levels of breaks saturate the assay and cannot be evaluated; in extreme cases, comet images disappear from the slides. However, high concentrations causing such effects are likely to be cytotoxic and so irrelevant for genotoxicity testing.

To summarise, the comet assay is an appropriate method for genotoxicity testing of ENMs, but only in combination with tests for cytotoxicity (so that genotoxicity can be related to concentrations above 80% viability). Treatment should be long enough for the ENMs to enter the cells: at least 24 h (and optionally 2–3 h) is recommended. Appropriate controls and reference standards should be included in tests; the solvent control and (if ENMs are coated) also the solvent plus coating material should be tested separately. Historical control data (for each cell type used) are valuable references for quality control. Genotoxicity tests must be accompanied by extensive characterisation of ENMs, in the test-medium (before and after exposure), and by tests for uptake by cells, since these factors have a marked influence on toxicity, including genotoxicity. When referring to concentrations of ENMs, cell number, cell culture plate format and volume of treatment medium on the plate are crucial factors that should be kept constant within the study, especially when comparing effects of different ENMs (8).

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