REVIEW

Confounding experimental considerations in nanogenotoxicology

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The development of novel nanomaterials with unique physico-chemical properties is increasing at a rapid rate, with potential applications across a broad range of manufacturing industries and consumer products. Nanomaterial safety is therefore becoming an increasingly contentious issue that has intensified over the past 4 years, and in response, a steady stream of studies focusing on nanotoxicology are emerging. However, it is becoming increasingly evident that nanomaterials cannot be treated in the same manner as chemical compounds with regards to their safety assessment, as their unique physico-chemical properties are also responsible for unexpected interactions with experimental components that generate misleading data-sets. In this report, we focus on nanomaterial interactions with colorimetric and fluorometric dyes, components of cell culture growth medium and genotoxicity assay components, and the resultant consequences on test systems are demonstrated. Thus, highlighting some of the potential confounding factors that need to be considered in order to ensure that in vitro genotoxicity assays report true biological impacts in response to nanomaterial exposure.

Introduction

Nanogenotoxicology is a sub-discipline that has recently emerged as a result of the dramatic expansion of the nanotechnology industry, which is continuously developing new nanomaterials at a rapid rate. Although we have been exposed to particles on the nanoscale for centuries through environmental sources such as volcanoes, viruses, dust storms and, more recently, increasing air pollution, engineered nanomaterials represent a new and largely undefined risk (1–3). Nanomaterials are defined as substances with at least one dimension <100 nm. They come in a myriad of different forms – 1900 different types of nanomaterials have been designed and fabricated, each of which with distinct physico-chemical features (Nanowerk Nanomaterial Database™). They range in their dimensions, shape (spheres, rods, cages, fibres, tubes) and composition (individual metals, metal oxides, binary metals or more complex combinations), with further intricacies introduced through the vast array of surface modifications (e.g. dextran, polyethylene glycol, DNA, amines, carboxyl groups, proteins) to suit specific applications. Consequently, nanomaterials are promising to revolutionize our lifestyles by improving a wide range of industrial, consumer and medical health care products. Some nanomaterials such as carbon nanotubes have very high tensile strength but are light and therefore pose many advantages over the use of more traditional metals in the automotive and aerospace industries (12). Due to their large surface area, nanomaterials often have unusual catalytic properties that have demonstrated great promise in improving propellant and fuel catalysts, automotive catalytic converters and also in environmental remediation where they will readily react with pollutants to detoxify them (13,14). Other applications include nanoelectronics (enhanced monitor resolution, high-energy batteries), but more direct human contact is likely to come from the personal care and medical health care products that are being developed. Titanium dioxide and zinc oxide nanoparticles can be found in suncream, while anti-ageing creams and cosmetics such as lipstick are now also being manufactured with nanoparticles as a component. Nanomaterials are not currently being used in the clinic, but recent studies have found that they have the potential to be applied to the development of novel tissue scaffolds, as nanomedicines, intelligent drug delivery systems able to target specific diseased cells and in improved non-invasive techniques for enhancing medical imaging capabilities (15,16). One material in particular that is already starting to leave an impression within the clinical arena is ultrafine superparamagnetic iron oxide nanoparticles (USPION). These nanoparticles have been found to substantially increase the contrast of magnetic resonance imaging, but can also be used as targeted drug delivery vehicles (by localizing magnetic particles carrying drugs to specific sites using magnetic fields) and in the destruction of tumour tissue report that many nanomaterials do indeed induce cytotoxicity, oxidative stress and inflammatory responses, but there are many uncertainties and conflicts in the literature (8–11). Given the expected consumer, occupational and clinical exposure scenarios predicted in the future, it is therefore imperative that we understand and thereafter minimize any safety hazards associated with nanomaterials, not only to protect human health and the environment but also to avoid damaging the nanotechnology industry in the long term.
through magnetic tumour ablation, known as hyperthermia (17).

Clearly, if the future applications of nanomaterials are realized, there is likely to be extensive occupational, consumer and clinical exposure. Primary routes of uptake into the body will be via the respiratory tract, gastrointestinal tract or through the skin, but injection or implantation of novel nanomedicines, tissue replacements or imaging agents represent an alternative entry route into the body. Thus, there is clearly a need for an understanding of the toxicological implications associated with exposure to engineered nanomaterials, but a complication that sets them aside from more conventional chemical or pharmaceutical safety testing is the necessity for complete physico-chemical characterization alongside their genotoxicological assessment (18). This is an essential consideration as the specific physico-chemical parameters of nanomaterials are responsible for creating their novel characteristics that enable the above described applications plus many more, but these same features may also govern unexpected biological interactions.

An obstacle that is now becoming increasingly evident is that traditional genotoxicological assays have been standardized and optimized for chemical compounds and we cannot assume that nanomaterials can be tested in the same way. Due to their size, nanomaterials have large surface areas as the particle number per unit weight is substantially higher than if they were micron sized. This characteristic provides them with particularly high reactivity because surface atoms usually have unsatisfied high-energy bonds that will readily interact with other molecules to become stabilized (19). Thus, nanomaterials are able to readily adsorb a wide variety of organic molecules and macromolecules on to their surface, which may influence the results of in vitro experiments in particular if adequate measures to assess these interactions have not been applied.

Given the reactivity of nanomaterials, assay compatibility is a very important consideration, which if not given enough credence, can have a dramatic impact on the validity of resultant data-sets and will therefore be the focus of this report. We will review and demonstrate some of the hurdles that may be encountered with in vitro testing systems in the form of nanomaterial interactions with experimental components, drawing attention to potential sources of error that would unwittingly generate misleading results. Many of these factors are only just coming to light, and thus are likely to account for some of the inconsistencies in the current literature, which contribute to the difficulty in reaching firm conclusions on the safety of nanomaterials. Accounting for such confounding factors within the experimental system when initiating nanogenotoxicology investigations will therefore improve the quality of in vitro assays, providing more reliable and reproducible data-sets.

**Colorimetric and fluorometric dyes**

Central components in a range of assays that compliment genotoxicity testing are colorimetric and fluorescence dyes that are capable of providing additional information such as quantification of cell viability or indicators for mechanisms of action. In many of these test systems, accurate and reproducible quantitation of colorimetric or fluorescence absorption and emission variations at specific light wavelengths are critical. Thus, if the test substance has the capacity to alter the optical properties of these dyes, the resultant data-sets will be inaccurate and could subsequently lead to flawed interpretations that may have a heavy influence upon safety assessment exercises.

Colorimetric and fluorometric dyes are proving to be problematic when incorporated into assays along with nanomaterials as there is increasing evidence that certain nanomaterials interact with these agents leading to false absorbance results. For example, several studies have found that carbon-based nanomaterials and carbon nanotubes interact with both fluorometric and colorimetric dyes in a range of cell viability assays including the 3-(4,5-dimethylthiazole-2-yi)-2,5-biphenyl tetrazolium bromide (MTT), neutral red, alamar blue, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) and Coomassie blue assays, leading to unreliable results (20–26). Furthermore, carbon nanotubes have been found to interact with organic dyes and the pH indicator (phenol red) often incorporated in tissue culture growth medium, altering their absorption or fluorescence spectra (27–30). The precise mechanisms involved in many of these interferences are not well understood, but carbaceous material is notorious for its ability to adsorb organic molecules and macromolecules from aqueous solutions through van der Waals forces. It is therefore likely that this effect is exacerbated when they are reduced to the nanoscale because of their increased surface area. Carbon nanomaterials therefore have the capacity to adsorb dyes onto their surface, which would subsequently quench or alter their fluorescence or absorbance properties and this has indeed been observed in a number of studies (21,22,25,26). However, this scenario is simplistic and factors such as surface chemistry, fabrication process or the types of surfactants used to disperse the nanomaterials also appear to play a role in governing the interactions and degree of interference with colorimetric and fluorometric dyes (20).

These observations are not only limited to carbon-containing nanomaterials. Silver nanoparticles have been found to cause spectral changes when they interact with fluorescent dyes, specifically altering emission intensity and in some cases quenching them altogether (31). We have also found in our studies that dextran-coated USPION interfere with the 3-(4,5-dimethylthiazole-2-yi)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell viability assay. This test is based on the reduction of the MTS salt to a soluble formazan product by cellular metabolism, the absorbance of which is measured at 490 nm and thus is directly proportional to the number of live cells. It is a simple method for the sensitive measurement of cell viability and thus has been adopted in a number of studies for the assessment of cytotoxicity induced by zinc oxide, titanium dioxide and iron oxide nanoparticles (32,33). However, we have found that when USPION are mixed with the necessary components of the MTS assay in a cell-free system, absorbance readings at 490 nm are dramatically increased (Figure 1), thus compromising its ability and sensitivity to accurately measure cell viability in response to USPION exposure. Hence, preliminary experiments on all colorimetric assays are necessary to identify any artifactual sources and in some cases there may be a need to assess cytotoxicity with more than one test system or avoid assays using coloured or fluorescent reagents in favour of direct cell counting.

In addition to cell viability assays, fluorescence-based dyes are key reporters for oxidative stress, which is particularly problematic as this is one of the primary mechanisms
associated with negative cellular responses to nanomaterials. Of the wide ranging and diverse methodologies and techniques available for the detection and measurement of oxidative stress, most assays fall into one of three main categories: (i) quantitation of oxidative species, (ii) determination of the degree of oxidative damage to proteins, DNA or lipids and (iii) assays that quantify levels of antioxidants. The only direct way of measuring specific reactive oxygen species (ROS) is through electron spin resonance (ESR; also called electron paramagnetic resonance). Pacurari et al. (34) used this technique for investigating ROS production induced by human mesenchymal cells exposed to single-walled carbon nanotubes (SWCNT), but there is currently no information on whether or not ESR is subject to interference by nanomaterials. However, this technique is not readily available in most laboratories and thus is not as frequently used for detecting nanoparticle-induced oxidative stress as other techniques such as those based upon fluorometric dyes, which are both convenient and sensitive. An example of a widely used probe for the detection of oxidative stress is the fluorometric dye 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) (35,36). This probe has been used extensively for quantitating nanomaterial-induced ROS in a range of cell types, some examples of which include iron oxide nanoparticles exposed to mesenchymal stem cells and in HeLa (human cervical carcinoma) cells (37); SWCNT-induced ROS in HaCaT (human keratinocyte) cells (38); ROS induced by ultrafine carbon black in the J774 (mouse macrophage) cell line (39) and by ambient ultrafine particles, cationic polystyrene nanospheres, TiO2 and fullerol nanoparticles in RAW 264.7 phagocytic cells (40).

Initiation of the DCFH-DA assay requires the diacetate portion of the molecule to be cleaved. In acellular systems, this can be achieved by chemical means (e.g. using sodium hydroxide) or through media-induced cleavage (41). However, this dye is most commonly used to detect intracellular ROS, as the hydrophobic DCFH-DA molecule readily penetrates the cell membrane, and then the diacetate portion is enzymatically cleaved by ubiquitous intracellular esterases. The non-fluorescent DCFH is subsequently oxidized by ROS to its highly fluorescent product 2′,7′-dichlorofluorescein (DCF) (42,43). Excitation of the DCF molecule at 485 nm emits green fluorescence at levels proportional to the amount of ROS present, which can be detected at 520 nm by fluorescence microplate readers, flow cytometry or confocal microscopy. However, DCFH has an unstable nature. It is slowly oxidized in air to DCF and is also prone to photo-oxidation by the laser light utilized for fluorescence microscopy. Thus, detection of intracellular fluorescent DCF is prone to false-positive results (35,44).

Another consideration when using DCFH-DA for quantifying nanomaterial induced oxidative stress, as with all fluorometric and colorimetric dyes is the possibility of their direct interaction with the probe, which can also lead to misguided interpretations. Indeed, we have found that in a cell-free system, dextran-coated iron oxide nanoparticles were able to interfere with the fluorescence emission of DCFH, the degree of which varied according to the concentration of the dye and the oxidation state of the nanoparticles (Figure 2). The interference was more pronounced with Fe3O4 than Fe2O3 nanoparticles, the former of which caused a significant dose-dependent decrease in fluorescence emission at both concentrations of dye tested, suggesting that the nanoparticles may be quenching the fluorescence response, possibly through adsorption onto their surface. In contrast, where interference was observed with Fe3O4, at very low doses, the fluorescence signal was slightly decreased and then increased in a dose-dependent manner after 0.1 µg/ml. The mechanism underlying this pattern of interference with the fluorescence response is currently unknown. However, a possibility is that Fe3O4 is able to quench fluorescence response at low concentrations (as Fe2O3 does), but it might have a higher oxidative potential than Fe3O4.

**Fig. 1.** Consequence of USPION in varying concentrations of serum, on the absorbance at 490 nm of the Cell Titre® Aqueous Non-Radioactive MTS cell viability assay (Promega, Southampton, UK) performed according to the manufacturers’ instructions, but in a cell-free system.
nanoparticles, such that at higher concentrations, their oxidative ability outweighs their fluorescence quenching potential, resulting in an overall increase in fluorescence response. Thus, with regards to DCFH-DA, potential interactions with nanoparticles need to be considered if the investigators wish to reliably use the assays in a quantitative manner.

DCFH-DA detects a wide range of oxidative species as DCFH can be oxidized by RO2•, RO•, OH•, HOC1 and ONOO (but not O2• and H2O2), hence its popularity. However, in some cases, an investigator may wish to detect a specific ROS species. There are therefore a number of alternative fluorescence probes for the detection of ROS and an example that has been specifically used to assess oxidative stress induced by SWCNT in RAW 264.7 macrophages is dihydroethidine (DHE) (45). This assay is capable of detecting superoxide, which oxidizes DHE to the fluorescent product 2-hydroxyethidium that intercalates into nuclear DNA, to emit strong fluorescence (46). However, as yet, there is no evidence to indicate how reliable these alternative dyes are when incorporated into experimental systems containing nanomaterials.

For assays that utilize colorimetric or fluorometric dyes, it is therefore critical to consider and control for the possibility that the test nanomaterial may react with the detection method. As we have seen, even subtle differences in oxidation state can have very different impacts on resultant data-sets. Thus, such interference will need to be assessed on a nanomaterial-by-nanomaterial basis until we fully understand the physico-chemical properties underlying these interactions.

**In vitro culture medium components**

Unlike the dyes mentioned in the previous section, cell culture underpins all *in vitro* genotoxicity tests and thus cannot be avoided. Consequently, interactions between nanomaterials and growth medium components will almost certainly impact on any subsequent genotoxicity test results. Such potential interactions therefore need to be identified, and where possible avoided or controlled for to ensure the reliability of subsequent data-sets.

Culture medium contains an array of growth factors, proteins and nutrients necessary to support cell growth, but biomolecules are readily adsorbed onto the surface of nanomaterials forming what is now referred to as a nanomaterial–protein corona (47). The density and composition of this adsorbed protein layer are likely to be dependent upon the make-up of the aqueous or physiological environment, in combination with the physico-chemical features of the nanomaterial itself, thus the corona is likely to consist of a dynamic and complex mixture of proteins. This protein coat would be directly in contact with cells during dosing regimes, as it essentially masks the nanomaterial. Hence, recent evidence indicates that the protein corona is responsible for governing uptake and intracellular location, thereby modulating cellular responses to nanomaterial exposure (47,48).

In support of the importance of considering the protein corona, a number of reports in the literature have indicated that serum content in cell culture medium can influence nanomaterial uptake into cells, but it is both cell type and nanomaterial dependent. For example, serum is responsible for decreasing the cellular uptake of silica-coated nanoparticles and anionic maghemite nanoparticles in HeLa cells, but uptake of the latter nanoparticles by macrophages is promoted by serum (49,50). We have observed in our own studies that serum negates the uptake of dextran-coated USPION by human lymphoblastoid B-cells (MCL-5) as illustrated in Figure 3. This negative effect of serum on nanoparticle uptake may be the result of an increased hydrodynamic diameter or altered electrostatic charges following adsorption of the serum proteins onto the surface of the nanomaterials. This would reduce the ability of these nanomaterials to enter cells by endocytosis or diffusion, while making them a more prominent target for phagocytosis by macrophages. Serum interactions with nanomaterials therefore have the potential to weigh heavily on the resultant biological impact of exposure to these substances where genotoxicity assays are concerned. For example, the presence of 10% serum in the culture medium during exposure of human lung epithelial cells to SWCNT substantially reduces the level of micronuclei (corresponding to chromosomal damage) induced (Figure 4).

In contrast, amorphous silica nanoparticles are more toxic to RAW 264.7 macrophage cells in the presence of serum than when dispersed using the surfactant Pluronic F127, possibly as the protein–nanoparticle interactions govern cellular uptake (54). However, in this same study, no such effect was seen with SWCNT, highlighting the dependence upon the physico-chemical characteristics of the nanomaterial itself in influencing the adsorption of proteins onto their surface and the resultant cellular consequences.

A conflicting observation is that serum acts as a surfactant to disperse nanomaterials, which are inherently hydrophobic and this has been reported in numerous studies with a range of nanomaterials including SWCNT, metals and metal oxide nanoparticles (27,55–59). Indeed, we have observed ourselves such an effect with dextran-coated USPION, where the hydrodynamic diameter of the agglomerated nanoparticles is lower in medium containing 10% serum as compared to 1% serum (Figure 5), which could be attributed to destabilization of the aggregates by the serum proteins. However, with increasing concentration, the aggregate size becomes larger and the degree of dispersal caused by 1% versus 10% serum becomes less pronounced, which has also been noted with several other nanoparticles (56,57). Hence, size measurements at a single concentration does not provide sufficient information on the experimental dynamics of nanomaterial agglomeration. Given that size distribution and agglomeration will impact the capacity for cellular uptake and subsequently their genotoxic potential, they should be assessed over a range of appropriate doses under the experimental conditions utilized. Thus, in nanogenotoxicity studies, it is becoming increasingly apparent that physico-chemical features must be characterized under the experimental setting so that definitive associations between these parameters and any biological responses observed may be identified. There are, however, difficulties in monitoring nanomaterial behaviour when dispersed in physiological solutions as the latter often contain particulate and charged materials that will mask the true size distribution and charge measurements of the nanomaterials themselves. Agglomeration can also be temperature dependent and so measurements should be made at 37°C, which requires temperature-controlled equipment. Additionally, many of the techniques currently available to assess surface area, morphology and composition are reliant on dry samples and so are difficult to apply to nanomaterials in solution. Novel or adapted technologies therefore need to be developed to enable
physico-chemical characterization of nanomaterials under these complex conditions.

Furthermore, there is evidence that the culture medium components themselves may be involved in cellular toxicity through nanomaterial interactions. For example, in addition to serum, SWCNT have been found to interact with pH indicators and riboflavin (vitamin B2) in growth medium, reducing the effective concentrations of nutrients in the cultures and thereby facilitating unfavourable growth conditions which could extend...
to an indirect toxic effect (60). This study also highlighted that the level of interaction observed was dependent upon the fabrication methodology used to synthesize the SWCNT with high-pressure carbon monoxide conversion nanotubes demonstrating increased interaction with medium components as compared to nanotubes generated by the arc discharge method.

Thus, the effects of serum and culture media components clearly need to be established on an individual nanomaterial basis prior to toxicological evaluation. Most reports to date have focused on serum, with it acting as a dispersant in some cases, reducing the overall size of the nanomaterial exposed to the cells and hence facilitating cellular internalization, possibly via receptor-mediated endocytosis or simple diffusion across the membrane. While in other cases, the serum proteins adsorbed onto the surface of the nanomaterial actually hinder their uptake in certain cell types. It is therefore becoming apparent that to further understand the nature of cellular responses to nanomaterials, characterization of the dynamic protein–nanomaterial interactions and the resultant adsorbed corona that forms under experimental conditions is an important challenge to be addressed. However, the consequent impact is also dependent upon the chemical properties of the nanomaterial surface and thus re-enforces the requirement for thorough physico-chemical characterization in parallel with adequate controls to assess potential confounding factors within in vitro safety testing studies.

Genotoxicity assay components

Most genotoxicity assays rely on a large number of intrinsic reagents essential to the testing methods’ ability to report on a specific cellular response following exposure to an exogenous agent. Due to the inherent reactivity of nanomaterials, this proves to be a potential problem as the test materials may themselves interact with key assay components, rendering them unfit for their given purpose. An example we will address here is focused on the cytokinesis-blocked micronucleus (CBMN) assay.

The micronucleus assay is a sensitive quantitative measure of the degree of gross chromosomal damage induced by a test agent, which can subsequently be classified as clastogenic events (chromosomal fragmentation) or aneugenic damage (changes in chromosome copy number). However, this assay can be performed using a number of slightly differing methodologies as illustrated in Figure 6. The schematics in Figure 6A and B are referred to as the CBMN assay and rely on the use of cytochalasin B to inhibit cytokinesis, generating binucleated cells in which the micronucleus frequency is scored. Alternatively, the mononuclear micronucleus assay can be used, which does not require cytochalasin B. Often, the CBMN assay is the preferred method as it limits the scoring of cells to only those that have undergone mitosis in the presence of the test compound. However, this is potentially a problem with regards to the study of nanomaterials because cytochalasin B also inhibits endocytosis, which is an important cell uptake mechanism favoured by some nanomaterials. Indeed, we have found that the specific micronucleus assay methodology utilized is a very important consideration for ultrafine nanoparticles, where endocytosis plays a key role in facilitating their cellular internalization. As illustrated in Figure 7, a significant dose-dependent increase in micronuclei when MCL-5 cells are exposed to dextran-coated USPION is only observed when the CBMN assay is performed with sequential treatment with the nanoparticles, followed by cytochalasin B (i.e. as illustrated in Figure 6A). When the USPION and cytochalasin B are co-exposed, no increase in micronucleus frequency is induced over a wide dose range, suggesting that the nanoparticles are not becoming internalized due to the inhibition of endocytosis.

Interestingly, the mononuclear micronucleus assay also failed to detect any increase in genotoxicity, but this is likely to be due to the sensitivity of the assay, in that double the number of cells need to be scored to observe the same level of micronuclei. In the CBMN assay, micronuclei are scored in cells to only those that have undergone mitosis in the presence of the test compound. However, this is potentially a problem with regards to the study of nanomaterials because cytochalasin B also inhibits endocytosis, which is an important cell uptake mechanism favoured by some nanomaterials. Indeed, we have found that the specific micronucleus assay methodology utilized is a very important consideration for ultrafine nanoparticles, where endocytosis plays a key role in facilitating their cellular internalization. As illustrated in Figure 7, a significant dose-dependent increase in micronuclei when MCL-5 cells are exposed to dextran-coated USPION is only observed when the CBMN assay is performed with sequential treatment with the nanoparticles, followed by cytochalasin B (i.e. as illustrated in Figure 6A). When the USPION and cytochalasin B are co-exposed, no increase in micronucleus frequency is induced over a wide dose range, suggesting that the nanoparticles are not becoming internalized due to the inhibition of endocytosis.

Interestingly, the mononuclear micronucleus assay also failed to detect any increase in genotoxicity, but this is likely to be due to the sensitivity of the assay, in that double the number of cells need to be scored to observe the same level of micronuclei. In the CBMN assay, micronuclei are scored in cells that are prevented from completing division. Hence, it follows that when one micronucleus is scored in one binucleate cell, if that cell were allowed to continue to divide, the micronuclei would only be located in one of the subsequent daughter cells. Thus, only one micronucleus would be scored out of two mononuclear cells (61).

SWCNT have very different physico-chemical features as compared to ultrafine nanoparticles, so we also assessed the sensitivity of all three version of the micronucleus assay methodology to determine the most sensitive for this type of nanomaterial. The SWCNT assessed were synthesized by the HiPCO method, had lengths of 400–800 nm, a 1- to 2-nm diameter, 98% purity and a surface area of 585 m²/g. As can be seen in Figure 8, there were differences in the frequency of micronuclei reported by each of the three assays. When

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**Fig. 6.** Micronucleus assay methodologies. (A) Cytokinesis-blocked method, with sequential exposure to the test agent, followed by cytchalasin B treatment. (B) cytokinesis-blocked method with simultaneous exposure to the test agent and cytchalasin B treatment and (C) mononuclear micronucleus assay.
considering the CBMN assay with simultaneous exposure of cytochalasin B with 50 or 100 μg/ml SWCNT, significant increases in micronucleus frequency were observed. This suggests that endocytosis is not the only means for SWCNT uptake leading to DNA damage, as micronuclei were observed despite the co-incubation of both SWCNT and cytochalasin B. Nonetheless, these alternative mechanisms of uptake appear to reach saturation as the same frequency of micronuclei was present at the two highest doses, while with the other two versions of the assay a dose-dependent increase was observed. Endocytosis may therefore be at least partially involved, accounting for the continuing increase in DNA damage with dose in both the mononuclear and the sequential CBMN assays. Indeed, when the shape of the micronucleus frequency curve is considered, it is very similar for both the mononuclear and the sequential CBMN assays, providing firm evidence for the significant dose-dependent increase in chromosomal damage following exposure to these SWCNT. However, the frequency of micronuclei observed in the mononuclear assay is approximately half that detected by the sequential CBMN assay, as expected due to the nature of the scoring (as mentioned above).

Both dextran-coated UPSION and SWCNT therefore demonstrate genotoxicity at non-cytotoxic doses, but these examples clearly demonstrate that careful consideration is required when utilizing traditional methodologies to assess the genotoxicity of nanomaterials.

**In vitro exposure regime considerations**

An important parameter in any *in vitro* genotoxicological assay is the duration of exposure, which typically does not exceed 24 h for chemical compounds due to their capacity to diffuse into cells and the length of their half-lives. However, the uptake and movement of nanomaterials through cells are likely to be slower than chemical diffusion and thus longer exposure periods may be necessary for effects such as genotoxicity to develop. To date, there are very few studies that have considered treatment times >24 h, with exception of an investigation on cobalt nanoparticles where uptake at 48 h was double that seen at 24 h (62) and in support, we have also seen the same increased uptake with time for dextran-coated USPION (data not shown).

Fig. 7. Comparison of micronucleus assay data generated following exposure of MCL-5 human lymphoblastoid B-cells to dextran-coated UPSION for 24 h in the presence of reduced (1%) serum. All experiments were performed in duplicate, with scoring as described in ref. (53). (A) CBMN assay with simultaneous exposure of USPION and cytochalasin B, (B) CBMN assay with sequential exposure to USPION followed by cytochalasin B and (C) mononuclear micronucleus assay (*P < 0.05; Fisher’s exact test used to compare frequency of micronuclei in treated samples to the control where water was used in place of the diluted USPION exposure).

Fig. 8. Comparison of micronucleus assay data generated following exposure of BEAS-2B lung epithelial cells to SWCNT for 48 h in the presence of reduced (2%) serum. Immediately before application to the cells, SWCNT were dispersed in tissue culture media by sonication for 1 h at 4°C. All experiments were performed in duplicate, with scoring as described in ref. (53). CBMN Sim—CBMN assay with simultaneous exposure of SWCNT and cytochalasin B, CBMN Seq—CBMN assay with sequential exposure to SWCNT followed by cytochalasin B and Mn Mono—mononuclear micronucleus assay.
another study has attributed the pro-inflammatory effects of cobalt nanoparticles on endothelial cells, to the release of Co$^{2+}$ ions (64). Unfortunately, as most studies do not go >24-h exposure periods, or consider the state of the nanomaterials within the cells over extended periods of time as compared to their original characteristics, this data are currently lacking and further research is required to answer some of these questions.

Another consideration is dosimetry, which concerns quantifying dose in terms of the true quantity of nanomaterial internalized into a population of cells. Due to the inherent hydrophobicity of many nanomaterials, they often have a tendency to agglomerate in aqueous environments, which will largely negate the uptake of most of the administered dose into many non-phagocytic cell types, with only those particles that remain dispersed or as very small agglomerates becoming internalized. Yet, the degree of agglomeration is likely to vary with time during the exposure period due to the mechanics associated with alterations in surface chemistry of the nanomaterials. Furthermore, large agglomerates may sediment out of solution due to gravitational settling, the rate of which will vary according to their size, and thus, the overall exposure to adherent cell lines versus suspension cells could be very different. The dynamic evolution of nanoparticle dose in situ or ‘particokinetics’ will clearly impact cellular outcomes and needs to be considered to develop a detailed understanding of the factors that govern the genotoxic potential of engineered nanomaterials (65). However, the practical solutions to address these issues are currently lacking and will likely require the development of novel analysis methodologies as existing technology is unable to accommodate such applications.

Conclusions

It is becoming increasingly evident that nanomaterials cannot be treated in the same manner as chemical compounds with regards to their safety assessment as their unique physicochemical properties introduce an additional level of complexity. Until we have a comprehensive understanding of the mechanics underlying biological responses to nanomaterial exposure, it is of vital importance that the physical and chemical properties of the nanomaterials under study are extensively characterized and that full consideration is given to potential interactions with inherent experimental components that may give rise to misleading results. A multidisciplinary approach is therefore essential to ensure that as much information as possible is generated about the nanomaterials themselves, in addition to their dynamic properties under experimental conditions. The provision of this information together with well-validated (and where necessary adapted) test systems will be essential to enable the scientific community to make informed decisions upon the factors that govern the biological impact of exposure to nanomaterials and thus ultimately their safety.

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