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Can the Ames test provide an insight into nano-object mutagenicity? Investigating the interaction between nano-objects and bacteria

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Abstract

The aim of this study was to assess the interaction of a series of well characterised nano-objects with the Gram negative bacterium *Salmonella typhimurium,* and how such an interaction may relate to the potential mutagenicity of nano-objects. Transmission electron microscopy showed that nano-objects (Au-PMA-ATTO NPs, CeO_2 NPs, SWCNTs and MWCNTs), as well as CAFs entered *S. typhimurium*. Only DEPs did not penetrate/enter the bacteria, however, were the only particle stimulus to induce any significant mutagenicity through the Ames test. Comparison with a sophisticated 3D *in vitro* cell model showed CAFs, DEPs, SWCNTs and MWCNTs to cause a significant increase in mammalian cell proliferation, whilst both the Au-PMA-ATTO NPs and CeO_2 NPs had not significant adverse effects. In conclusion, these results indicate that various of different nano-objects are able to penetrate the double-lipid bilayer of Gram negative bacteria, although the Ames test may not be a good indicator for nano-object mutagenicity.

Keywords: nano-object(s), nano-bacterial interactions, mutagenicity, Ames test, mammalian cells

Introduction

Recently, the field of nanotoxicology (Oberdorster et al. 2005, 2007; Maynard 2007; Oberdorster 2010; Krug & Wick 2011) has highlighted the need for increased and in-depth genotoxic evaluation of both accidentally produced and engineered nano-objects (defined as a material with one, two or three external dimensions in the nanoscale (1–100 nm (ISO 2008))) (Doak et al. 2009; Singh et al. 2009; Donaldson et al. 2010). The need to fully understand the potential genotoxicity of nano-objects (Schins & Knaapen 2007; Landsiedel et al. 2009; Greim & Norppa 2010) has intensified through the findings of Poland et al. (Poland et al. 2008), who showed that carbon nanotubes (CNTs) (Iijima 1991), with multiple walls (MWCNTs) (BSI 2007), can cause increased granulomatous inflammation at the peritoneal aspect of the diaphragms' surface *in vivo*. The subsequent observation that this effect was also elicited by amosite asbestos fibres, and the conclusion that MWCNTs with a specific set of physico-chemical characteristics (Poland et al. 2008; Donaldson et al. 2010) can act similar to asbestos fibres raised extreme caution towards the use of such nanofibres (defined as a nano-object with two similar external dimensions in the nanoscale and the third dimension significantly larger, which can also be considered a high-aspect ratio nanoparticle (ISO 2008)) in their many proposed applications (Johnston et al. 2010; Wick et al. 2011).

Whilst the findings of Poland et al. (Poland et al. 2008) have been widely reported, the observations of a plethora of other studies focussing upon the genotoxicity of nano-objects have received less attention. Takagi and colleagues (Takagi et al. 2008) also showed that MWCNTs can cause reducing effects to the lung *in vivo*, reporting that MWCNTs induce mesothelioma formation *in vivo*. The dose applied to the mice in this study, however, has received increased criticism because the results reported can adequately be associated with an overload situation (Oberdorster 2010; Teeguarden et al. 2007). Similarly, the results of Ryman-Rasmussen et al. (Ryman-Rasmussen et al. 2009), who showed that the hazard of CNTs suggested by Poland et al. (Poland et al. 2008) is a real possibility because they were able to reach the pleural cavity of mice, should be taken with extreme caution due to the increased dose

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administered *in vivo*. Jacobsen et al. (Jacobsen et al. 2008; Jacobsen et al. 2008; Jacobsen et al. 2009) also reported that single-walled CNTs (SWCNTs) and C_{60} fullerenes can cause DNA damage *in vivo* and *in vitro*, although not to the extent elicited by diesel exhaust particles (DEPs). In addition, many other forms of nano-objects including titanium and zinc oxide (TiO_2 and ZnO) (Landsiedel et al. 2010) and other metal oxides (Grigg et al. 2009), as well as numerous other nano-objects (Lindberg et al. 2009; Pfaller et al. 2010), have been investigated as to their potential genotoxicity *in vivo* and *in vitro*. Discrepancies are paramount throughout such studies however, and any general overview, as necessary for hazard identification (Hansen et al. 2007), production of test guidelines (Warheit & Donner 2010) and scientific policy concerning the production and human exposure of nano-objects (Morris et al. 2011) is currently not possible. The equivocal nature of these studies can be, in part, attributed to (i) the different biological models employed, (ii) the dose/concentration administered, (iii) exposure method, (iv) duration of exposure and (v) the quality (e.g., purity) of the nano-objects used. Thus, it is evident that the potential detrimental (e.g., carcinogenic) effects associated with exposure to the wide variety of nano-objects currently available, and being produced must be understood. To achieve this, realisation of the advantages that nano-objects (nanotechnology) may pose must be gained, as well as the creation of adequate and efficient safety measures in both an occupational and consumer setting to restrict the short- and long-term exposure of nano-objects to humans and the environment (Maynard 2007; Maynard et al. 2006).

Increased efforts are being undertaken to understand both the realistic doses of nano-objects that humans could potentially be exposed to (Kuhlbusch et al. 2011), and to provide protocols for standardised physico-chemical characterisation of nano-objects (Bouwmeester et al. 2011). Despite this, the precise biological system necessary for gaining insight into the potential adverse effects of nano-objects is debatable (Morris et al. 2011; Maynard et al. 2006; Kuhlbusch et al. 2011; Bouwmeester et al. 2011; Rothen-Rutishauser et al. 2008; Hartung 2011). To try and satiate this issue, it is clear that reliable, efficient, reproducible biological (model) systems are needed (Rothen-Rutishauser et al. 2008; Hartung 2011). Whilst numerous *in vivo* and *in vitro* models are commonly used (Stone et al. 2009; Clift et al. 2011), other forms of biological systems are available. An example of this is bacteria, and specifically the bacterial-based 'Ames test' (Ames et al. 1973), an approved OECD mutagenicity testing method (OECD. 1997). Although bacteria are irrelevant systems for use in the assessment of the entry mechanism (s) of nano-objects (i.e., endocytosis) as it would occur in mammalian cells (i.e., the human body) (Stone et al. 2009), the benefits of the Ames test have led to it being proposed as a potentially advantageous model to be used to screen nano-object-associated mutagenicity. The main reason for this is that bacteria provide an advantageous system compared to *in vitro* cell cultures. Bacteria are (i) easy and quick to culture, (ii) robust (even more so than common eukaryotic cell cultures) and (iii) inexpensive. Additionally, bacterial culture methods require only a limited level of skill and equipment compared to mammalian cell culture (Madigan et al. 2008).

Compared to alternative mammalian genotoxic testing strategies that are commonly applied within nanotoxicology, such as the laborious and expensive micronucleus (Kirsch-Volders et al. 2011) and comet assays (Collins 2004; Barnes et al. 2008), the Ames test is cheaper and less laborious, and thus provides an ideal basis for the high-throughput screening (Aubrecht et al. 2007) of nano-objects for the reasons previously highlighted. However, debate surrounds the use of the Ames test for the testing of nano-object-related genotoxicity (Landsiedel et al. 2009; Greim & Norppa 2010; Stone et al. 2009; Jaurand et al. 2009; Ng et al. 2010). It is suggested that nano-objects, independent of their specific physico-chemical characteristics (i.e., solubility (Landsiedel et al. 2009; Greim & Norppa 2010; Stone et al. 2009; Jaurand et al. 2009; Ng et al. 2010)), are not able to penetrate the rigid outer double membrane of Gram negative bacteria, and/or are bactericidal (cause bacterial death). Thus, any mutagenic events observed are not nano-specific (Claxton et al. 2010). Furthermore, the OECD Working Party on Nanotechnology (WPN) (OECD) has been deliberating in recent years as to whether or not the Ames test is a relevant method for use in determining nanomaterial potential carcinogenicity.

So far, only a limited number of studies have used the Ames test, or alternative forms of bacteria (i.e., *Escherichia coli*) to assess the mutagenicity of nano-objects. Predominantly these studies have investigated the mutagenicity of DEPs (Zhou & Ye 1997; Bunger et al. 1998; Zhou & Ye 1998; Zhao et al. 2004; Singh et al. 2004), although other nano-objects (e.g., metal oxides and fullerenes) have also been assessed (Sera et al. 1996; Brayner et al. 2006; Kumar et al. 2011; Pan et al. 2010). Additionally, in the field of nano-ecotoxicology, due to their important role within various ecosystems, bacteria are regularly utilised as a model biological system (Behra & Krug 2008; Kahru & Dubourguier 2010). Commonly, a biological reaction is measured, and it is suggested that the nano-objects used are 'interacting' with the bacteria. However, closer interpretation suggests that the nano-objects used never (i) attach to the outer membrane or (ii) are located inside the bacteria. Such assumptions are also made in the studies in which the Ames test was used to measure the mutagenicity of various nano-objects (Zhao et al. 2004; Lin et al. 2008). It is essential therefore to understand the interaction of nano-objects with the biological system used; in this case bacteria. The aim of this study, therefore, was to assess various different nano-objects in regards to their effects as denoted by the Ames test (i.e., to determine the suitability of the test for understanding nano-object-associated mutagenicity), and to study the interaction with *Salmonella typhimurium* of a series of well-characterised and dispersed nano-objects (cerium dioxide (CeO_2) nanoparticles (NPs)) (defined as a nano-object with all three external dimensions in the nanoscale (1–100 nm) (ISO 2008)), Au-PMA-ATTO NPs, SWCNTs and MWCNTs. In addition, both crocidolite asbestos fibres (CAFs), a known pathogen, however, unknown entity in

regards to its bacterial interaction, and diesel exhaust particles (DEPs) as a positive mutagen control were used.

Methods

Chemicals and reagents

All chemicals and reagents were purchased from Xenometrix, Switzerland, unless otherwise stated.

Bacterial culture

S. typhimurium bacterial strains TA98 and TA1537 (base-pair substitution mutations), as well as TA100 and TA1535 (frame-shift mutations) were purchased as part of a diagnostic kit (Xenometrix AG, Switzerland) and stored at –70°C. Prior to culture, each bacterial strain was thawed at room temperature for 5 min. A total of 200 µl growth medium was then added to each vial. The dark, semi-solid bacterial pellet was then mechanically disrupted until a homogeneous bacterial suspension was obtained. For each bacterial strain, 25 µl of the bacterial suspension was added to 10 ml of growth medium (as supplied in the diagnostic kit by Xenometrix AG, Switzerland) in a sterile 50 ml tube. A total of 10 µl of Ampicillin was added to both the TA98 and TA100 strains, as well as the negative control (growth medium only). All strains were then incubated in an environmental shaker at 37°C, 250 rpm for 14–16 h. After the incubation period, the optical density (OD) value at 600 nm (OD_{600}) of each strain was determined. In a sterile, plastic cuvette, 100 µl of each bacterial strain was added to 900 µl growth medium. For subsequent experimental analysis to occur, each bacterial strain had to exhibit an OD_{600} of ≥ 2.0. The negative control was required to have an OD_{600} of ≤ 0.05. To use the TA100 bacterial strain, a subsequent incubation step was required. During this process, the bacterial strains TA98, TA1535, TA1537 and the negative control were stored at 4°C to maintain bacterial viability and proliferation status. In a fresh, sterile 50 ml tube, the TA100 strain was then diluted 1:4 with growth medium and incubated at 37°C, 250 rpm in an environmental shaker for 90 min. The OD_{600} was then measured as previously described. The new TA100 suspension required an OD_{600} of 1.5–1.9.

Nano-objects

The following nano-objects were investigated; (i) CeO_2 NPs, (ii) gold core/polymer shell with an embedded fluorophore (Au-PMA-ATTO) NPs (Lin et al. 2008), (iii) SWCNTs (Yangtze Nanotechnology, China) and (iv) MWCNTs (Cheap Tubes Inc., USA). In addition, reference materials of DEPs (NIST SRM #2975), as a positive mutagen control (DEPs are also defined as a human 1A carcinogen) and CAFs, a reference fibrous material, were also used. All nano-objects used have previously been described and characterised in Lin et al. (Lin et al. 2008) (Au-PMA-ATTO NPs), Raemy et al. (Raemy et al. 2011) (CeO_2 NPs) and Wick et al. (Wick et al. 2007) as well as Thurnherr et al. (Thurnherr et al. 2009) (SWCNTs, MWCNTs and CAFs). The physico-chemical characteristics of each different nano-object are summarised within Table I in supplementary information. The DEPs used in this study are reference materials produced by the National Institute for Science and Technology (NIST) (SRM #2975). All nano-objects and reference materials, except the Au-PMA-ATTO NPs, were assessed at 0.005, 0.01, 0.02, 0.03 and 0.04 $mg.mL^{-1}$. The Au-PMA-ATTO NPs were investigated at 3.125, 6.5, 12.5, 18.75 and 25 nM. All exposures were suspended in bacterial exposure medium (as supplied within the diagnostic kit by Xenometrix AG, Switzerland) prior to their exposure to the bacterial cultures.

Ames test

Briefly, the Ames test is based upon strains of *S. typhimurium* that have a mutation on the *HIS* operon, and thus are unable to generate the amino acid histidine that is essential for bacterial replication (Ames et al. 1973). The presence of a mutagenic agent, when exposed during the test, enables the bacteria to reverse the mutation on the *HIS* operon, resulting in histidine production and colony formation, also referred to as 'positive mutagenic events'. Commonly, the Ames test is applied with a combined extract (either from humans or animals), known as the 'S9 mix'. Deliberation still surrounds the use of this metabolic-extract fraction (Hakura et al. 1999) however, especially when concerning how a xenobiotic may affect (i.e., elicit carcinogenicity) alternative organs of the body, such as xenobiotics that are inhaled and not cleared from the lung (Roller 2009).

All bacterial overnight cultures were initially diluted 1:10 in bacterial exposure medium. In a 24 well-plate, 240 µl of bacterial culture was added and then exposed to10 µl of the nano-objects or reference materials. All samples were then incubated in an environmental shaker at 37°C, 250 rpm for 2 h. Following the incubation period, 2.8 mL of indicator medium (as per the diagnostic kit guidelines (Xenometrix AG, Switzerland)) was added to each well of the 24-well plate. A total of 50 µl of each sample was then transferred to a 384-well plate. Samples were then subsequently incubated for an additional 48 h in a dry incubator at 37°C. Following the 48 h incubation period, the 384-well plates were assessed for a colour change within each well, which, in line with the diagnostic kit, was indicative of histidine production by the *S. typhimurium* and resultant colony formation, and thus regarded as a 'positive event'. It is important to note that S9 liver extract was not used at any point in the study.

Nano-object:bacterial interactions

The interaction of each nano-object and reference material with the bacterial strain TA98 was investigated through conventional and elemental electron microscopy. The TA98 strain was cultured and exposed to each nano-object and reference material for 2 h as previously described. After the exposure period, samples were then fixed using 2.5% glutaraldehyde and then prepared for transmission electron microscopy (TEM) analysis as previously described in Brandenberger et al. (Brandenberger et al. 2010). All samples were imaged using conventional TEM (CM12 TEM (FEI Co. Philips Electron Optics, Zurich, Switzerland)) with subsequent elemental electron microscopy (Tecnai F20 TEM (FEI, Eindhoven, the Netherlands)) performed

upon the CeO_2 NP-exposed bacteria only, as previously described in Raemy et al. (Raemy et al. 2011).

Bacterial viability

Viability analysis was performed at 2 h and 48 h to mimic the interaction as it occurs in the Ames test. The viability of the TA98 bacterial strain was assessed both through CLSM and fluorescent spectroscopy using a Live/Dead *Bac*Light bacterial viability kit (Molecular Probes, Switzerland). The TA98 strain was cultured and exposed to each nano-object (and fibre/mutagen positive controls) as previously described.

Confocal laser scanning microscopy

After the exposure period, in the 24-well plate, all samples were exposed to 3 $\mu l.mL^{-1}$ of a combined volume solution of SYTO 9 (emission 500 nm) and propidium iodide (emission 635 nm). Samples were then incubated at room temperature with the combined fluorophore solution for 15 min, in the dark. A total of 25 µl was then applied to a microscope slide, with a cover slip then applied. Samples were then imaged through confocal laser scanning microscopy using a Carl Zeiss 510META (Carl Zeiss AG, Switzerland) and images were subsequently restored using IMARIS (Bitplane AG, Switzerland). In addition, it was possible to observe the presence of the Au-PMA-ATTO NPs in the fluorescently labelled bacteria, because these Au-PMA-ATTO NPs contain an embedded fluorophore (emission 543 nm) within their polymer shell (Lin et al. 2008). No spectral overlap was observed when imaging all fluorophores concomitantly (data not shown).

Fluorescent spectroscopy

After the exposure period, in the 24-well plate, all samples were exposed to 3 $\mu l.mL^{-1}$ of a combined volume solution of SYTO 9 (emission 500 nm) and propidium iodide (emission 635 nm). Samples were then incubated at room temperature with the combined fluorophore solution for 15 min, in the dark. A total of 25 µl was then placed into a 96-well plate, and samples were assessed using fluorescent spectroscopy at 490–700 nm (subtraction). Bacterial viability was determined by total relative fluorescent intensity after both 2 h and 48 h.

Mammalian cell culture

A sophisticated 3D *in vitro* triple cell co-culture model of the epithelial airway barrier (TCC-C) consisting of an epithelial cell layer (human bronchial epithelial cell-line 16HBE14o- (a generous gift from Dr. D. Gruenert (Cardiovascular Research Institute, University of California, San Francisco, USA))) and human whole blood monocyte-derived macrophages (MDM) and dendritic cells (MDDC) on the apical and basolateral sides, respectively, was used, in a 12-well plate (with membrane inserts) format, as previously described (Rothen-Rutishauser et al. 2008; Blank et al. 2007).

Assessment of mammalian cell proliferation

To assess the ability for the panel of nano-objects to effect mammalian cell proliferation, in comparison to the Ames test, CAFs, CeO_2 NPs, DEPs, SWCNTs and MWCNTs were exposed to the TCC-C at a concentration of 0.005, 0.01, 0.02, 0.03 and 0.04 $mg.mL^{-1}$, and the Au-PMA-ATTO NPS at 3.125, 6.5, 12.5, 18.75 and 25 nM for 48 h at 37°C, 5% CO_2. Cell samples were then assessed for their proliferative state through the EdU assay (a form of the BrdU assay) diagnostic kit (Invitrogen, Switzerland). Briefly, in a 12-well plate, the TCC-C (both upper and lower sections) was treated with 10 µM ErdU compound in PBS for 4 h. After this incubation period, samples were fixed with 3.7% paraformaldehyde in PBS for 15 min at room temperature, and then subsequently treated with 0.2% Triton X100 in PBS for 15 min at room temperature to permeablise the cell membrane for immunofluorescent staining to occur. To assess the impact of the different nano-objects upon cellular proliferation during the exposure period, an EdU cocktail solution (Invitrogen, Switzerland) containing Alexa Fluor 488 as a fluorescent marker for the DNA intercollating agent was prepared. Samples were incubated for 30 min at room temperature in the dark with the ErdU cocktail. Samples (upper and lower sections) were then combined prior to the fluorescent intensity being measured at 488 nm in a fluorescent spectrophotometer. As a positive control, mitomycin C and ethylmethanesulphonate were used as direct acting compounds, in addition to cyclophosphamide which is an indirect acting compound. All positive controls were used at 0.1 $mg.mL^{-1}$. Cell culture media (Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal calf serum, 1% L-Glutamine and 1% Penicillin/Streptomycin) acted as a negative control.

Statistical analysis

All results are presented as the mean ± standard error of the mean (SEM). All data was normally distributed (data not shown). Statistical significance of the Ames test data sets was determined through the twofold rule (Ames et al. 1973; Mortelmans & Zeiger 2000). This rule states that all data sets that are ≥ 50% of the negative control levels (positive mutagenic events) are significantly mutagenic. Quantitative viability and mammalian cell proliferation data sets were determined via a Student's t-test (MINITAB®, version 15.1, MINITAB Inc., 2006). The result of the Student's t-test was considered significant if $p \leq 0.05$.

Results

Ames test

Investigation of the ability for the series of different nano-objects tested in this study to elicit a mutagenic response by the Ames test showed that the TA98 strain (frame-shift mutation) of *S. typhimurium* elicited the strongest mutagenicity (Figure 1A–B). Despite this, only DEPs caused any significant mutagenicity (Figure 1A), as per the 'two-fold' rule (i.e., a synergistic positive mutagenic reaction) (Stone et al. 2009; Zhao et al. 2004). Although significant differences (through the twofold rule) can be observed for all nano-objects tested in both the TA1535 (base-pair mutation) and TA1537 (frame-shift mutation) strains (Figure 1A–B), these values are below the detection limit and within the level of variation known for the form of the Ames test

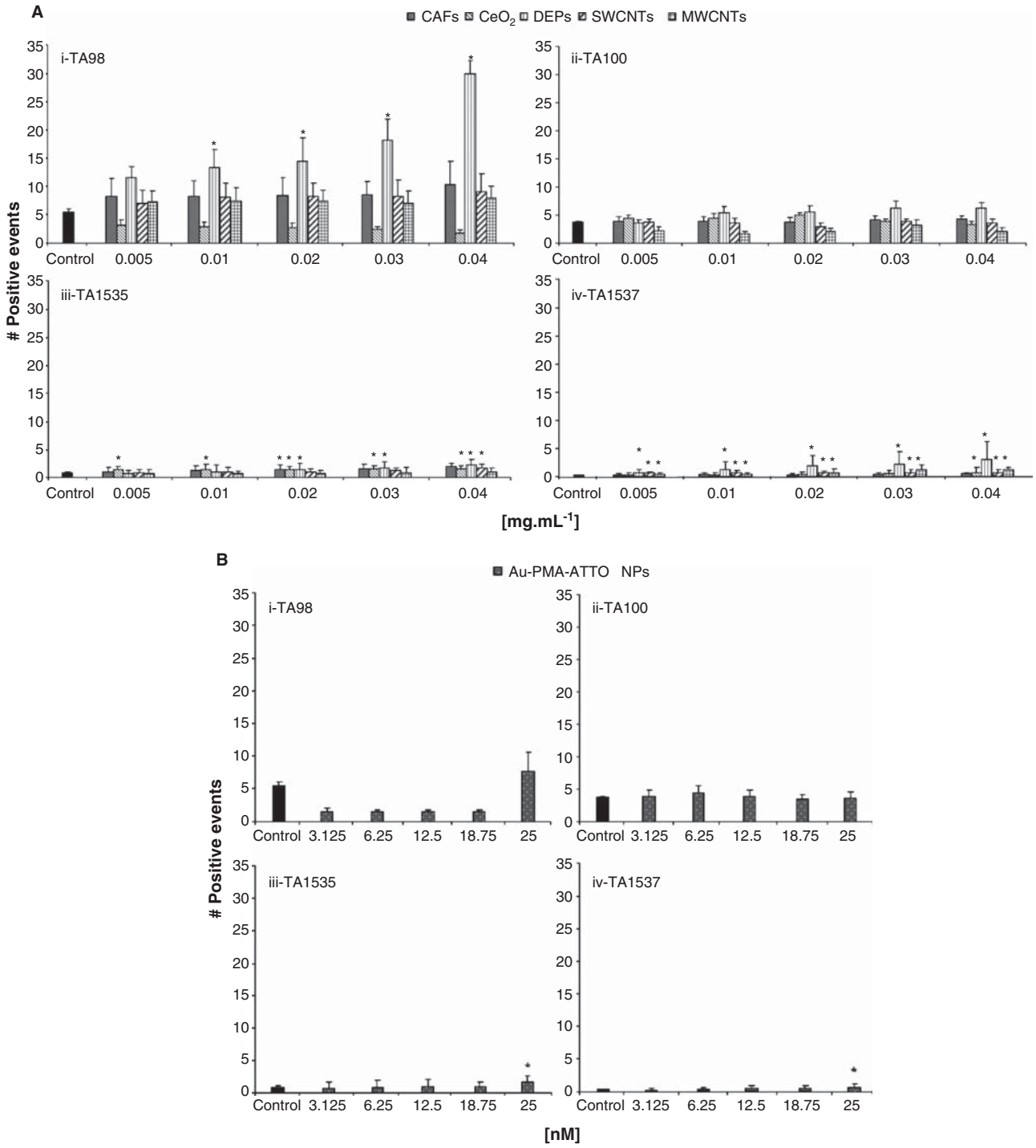

Figure 1. (A) Positive mutagenic events (Ames test) of the *Salmonella typhimurium* strains (**i**) TA98, (**ii**) TA100, (**iii**) TA1535 and (**iv**) TA1537 after 2 h exposure in a shaking incubator at 37°C and 250 rpm to crocidolite asbestos fibres (CAFs), cerium dioxide (CeO_2) nanoparticles (NPs), diesel exhaust particles (DEPs), single-walled and multi-walled carbon nanotubes (SWCNTs and MWCNTs) at 0.005, 0.01, 0.02, 0.03 and 0.04 mg.mL^{-1} and a subsequent 48 h post-incubation in a dry incubator at 37°C. Control values are the effects of the bacterial exposure media alone. Data is presented as the mean ± standard error of the mean (SEM) (n = 3). Statistical significance was determined using the 'two-fold' rule for the Ames test (Stone et al. 2009; Sera et al. 1996). (B) Positive mutagenic events (Ames test) of the *Salmonella typhimurium* strains (**i**) TA98, (**ii**) TA100, (**iii**) TA1535 and (**iv**) TA1537 after 2 h exposure in a shaking incubator at 37°C and 250 rpm to Au-PMA-ATTO nanoparticles (NPs) at 3.125, 6.25, 12.5, 18.75 and 25 nM and a subsequent 48 h post-incubation in a dry incubator at 37°C. Control values are the effects of the bacterial exposure media alone. Data is presented as the mean ± standard error of the mean (SEM) (n = 3). Statistical significance was determined using the 'two-fold' rule for the Ames test (Stone et al. 2009; Sera et al. 1996).

employed in this study (Blank et al. 2007). In contrast, no significant effects were found with any nano-object used in the other frame-shift mutation strain investigated; TA100 (Figure 1A–B).

Nano-object:bacterial interaction

Because the TA98 *S. typhimurium* strain is the most commonly used strain for the Ames test (Zhou & Ye 1997; Bunger et al. 1998; Zhou & Ye 1998; Zhao et al. 2004;

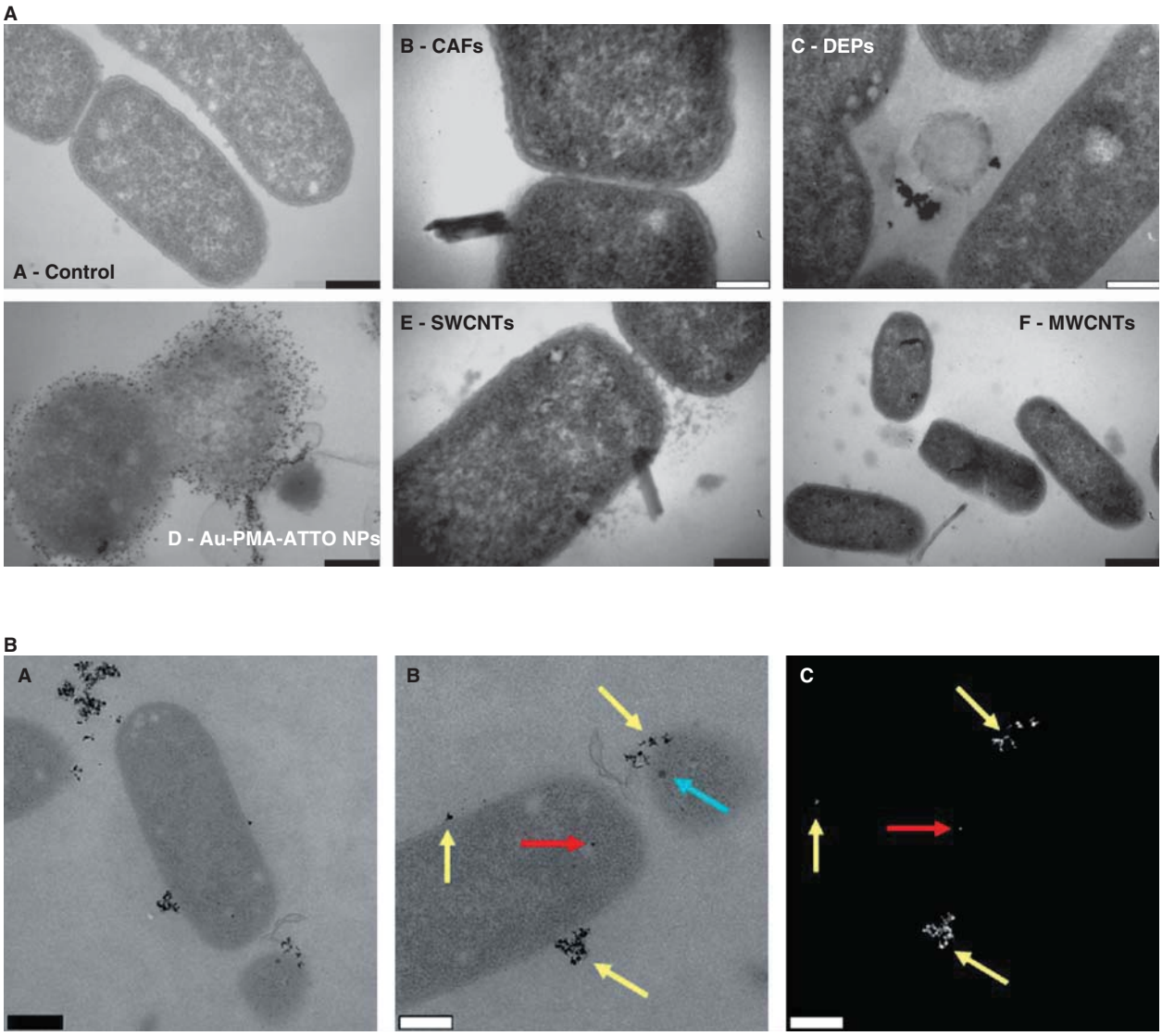

Figure 2. (A) Conventional transmission electron micrographs (TEM) of the interaction of (**A**) bacterial exposure media only, (**B**) crocidolite asbestos fibres (CAFs), (**C**) diesel exhaust particles (DEPs), (**D**) Au-PMA-ATTO NPs (25 nM), (**E**) single-walled and (**F**) multi-walled carbon nanotubes (SWCNTs and MWCNTs) with the TA98 *Salmonella typhimurium* strain following exposure at 0.04 mg.mL^{-1} for 2 h in a shaking incubator at 37°C and 250 rpm. Black and white scale bars represent 500 nm and 200 nm, respectively. (B) Transmission electron micrographs of the interaction of cerium dioxide (CeO_2) nanoparticles (NPs) with the TA98 *Salmonella typhimurium* strain following exposure at 0.04 mg.mL^{-1} for 2 h in a shaking incubator at 37°C and 250 rpm. Images **A** and **B** show the TA98 bacterial strain through conventional transmission electron microscopy. Image **C** shows the same field of view as in image **B**, however using electron spectroscopy imaging (ESI), previously described in Brandenberger et al. (Kumar et al. 2011), for CeO_2. In images **B** and **C**; **red** arrows highlight cerium present inside TA98 bacteria, **yellow** arrows indicate cerium that is either outside the bacteria or attached to the outside of the TA98 bacterial membrane, and **blue** arrows represent an example of TEM sample staining contamination. Black and white scale bars represent 500 nm and 200 nm, respectively.

Singh et al. 2004; Sera et al. 1996; Brayner et al. 2006; Kumar et al. 2011; Maenosono et al. 2007), and it was the only strain in this study to show any detectable mutagenicity, it was used for all subsequent investigation of the nano-object:bacterial interaction. Interestingly, it was observed that all (nano)fibres (SWCNTs, MWCNTs and CAFs) directly penetrated the membrane of the TA98 strain after a 2 h exposure period at 0.04 mg.mL^{-1} (Figure 2A). Similarly, investigation of the CeO_2 NP-bacterial interaction through elemental transmission electron microscopy (Brandenberger et al. 2010) showed these metal oxide NPs to be present on the membrane and inside the TA98 strain after 2 h at the same concentration as the (nano)fibres (Figure 2B). Interestingly, the Au-PMA-ATTO NPs, when exposed to *S. typhimurium* at 25 nM, always observed within an aggregated form, were noted to surround the TA98 bacterial strain, seemingly dissolving the lipid bilayer and potentially altering the morphology of the bacteria (Figure 2A). DEPs, however, were not found to be present inside the bacteria, or interaction with the bacterial membrane directly after 2 h at 0.04 mg.mL^{-1} (Figure 2A).

Bacterial viability

It is important to highlight that none of the nanofibres , as previously shown by Liu et al (2009), or the CeO_2 NPs caused any significant ($p > 0.05$) cytotoxicity to the TA98 strain over a 48 h period up to 0.04 mg.mL^{-1} (Figure 3A).

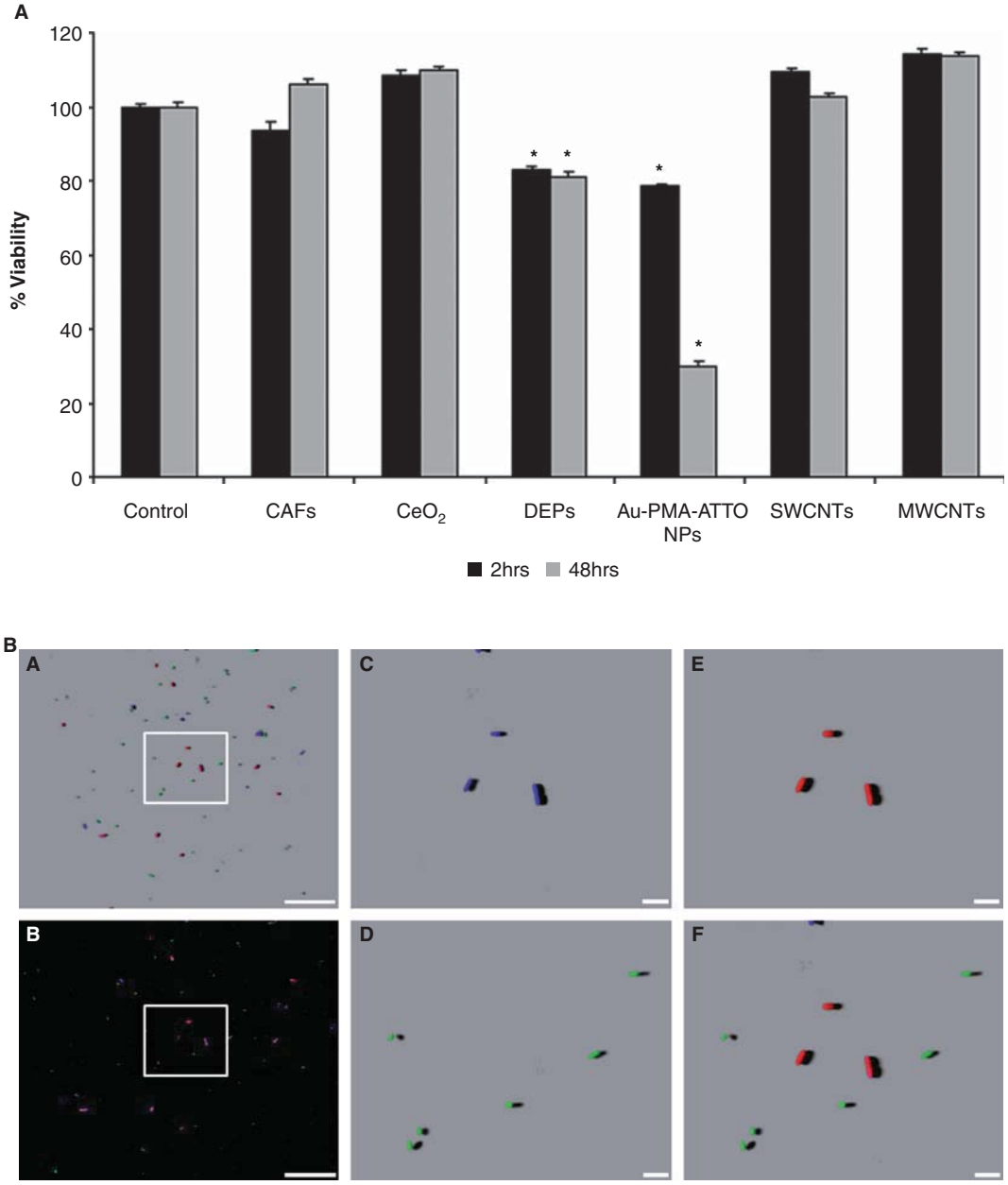

Figure 3. (A) Quantitative cytotoxicity of the TA98 strain of *Salmonella typhimurium* after exposure to crocidolite asbestos fibres (CAFs), cerium dioxide (CeO_2) nanoparticles (NPs), diesel exhaust particles (DEPs), single-walled and multi-walled carbon nanotubes (SWCNTs and MWCNTs) at 0.04 mg.mL^{-1}, as well as Au-PMA-ATTO NPs at 25 nM for 2 h in a shaking incubator at 37°C and 250 rpm and 48 h post-exposure in a dry incubator at 37°C. Data is presented as the mean ± standard error of the mean (SEM) ($n = 3$). Statistical significance was determined using a Student's t-test ($p < 0.05$). (B) Qualitative cytotoxicity of the TA98 strain of *Salmonella typhimurium* after exposure to Au-PMA-ATTO nanoparticles (NPs) at 25 nM for 2 h in a shaking incubator at 37°C and 250 rpm and 48 h post-exposure in a dry incubator at 37°C. Image **A** shows a representative field of view of an Au NP-exposed sample. Image **B** shows the representative field of view as observed prior to image reconstruction. The selected area (**bold** white box) is shown in images **C**–**E**. Image **C** shows dead (non-viable) TA98 bacteria, **D** shows the viable bacterial fraction and **E** shows bacteria containing Au-PMA-ATTO NPs. Image **F** shows the overlay of images **C**–**E**. The white scale bars represent 20 μm (**A** and **B**) and 5.5 μm (**C**–**E**), respectively.

DEPs, however, did elicit a significant ($p < 0.05$) decrease in bacterial viability over a 48 h period (Figure 3A). Whilst DEPs caused a significant, 20% decrease in bacterial viability, Au-PMA-ATTO NPs were found to significantly decrease ($p < 0.05$) viability by 70% after 48 h exposure (Figure 3A). Interestingly, analysis using confocal laser scanning microscopy showed that only the non-viable bacteria contained Au-PMA-ATTO NPs, and that the Au-PMA-ATTO NPs did not interact with any viable bacteria (Figure 3B).

Mammalian cell proliferation

After the 48 h exposure period, it was observed that CAFs, DEPs, SWCNTs and MWCNTs all showed a significant ($p < 0.05$) concentration-dependent increase in the proliferative state of the triple cell co-culture system (Figure 4A) from 0.005 to 0.04 mg.mL^{-1}. CeO_2 NPs, however, showed a limited effect upon cell proliferative state over the concentration range tested (0.005 to 0.04 mg.mL^{-1}), although no significance ($p > 0.05$) compared to baseline levels was

recorded (Figure 4A). Similar findings were also observed for the Au-PMA-ATTO NPs for the concentrations 3.125 to 25 nM. No significant ($p > 0.05$) changes in the cellular proliferative state of the 3D *in vitro* model was observed following the 48 h exposure period to these plasmonic NPs (Figure 4B).

Discussion

Increased debate surrounds the use of the 'Ames test' as a valid alternative method for determining nano-object-associated mutagenicity. The aim of this study therefore, was to assess the potential mutagenic capability of a series of well-defined nano-objects via the Ames test, and furthermore, to study how these different nano-objects interact with *S. typhimurium*.

The finding that DEPs cause significant mutagenicity when assessed by the Ames test supports previous literature that has shown DEPs to be mutagenic when produced from various different engine types (Zhou & Ye 1997; Bunger et al. 1998; Zhou & Ye 1998; Zhao et al. 2004; Singh et al. 2004; Belisario et al. 1984; Wallace et al. 1987). In this study, a complete DEP reference material sample was used (NIST SRM 2975) without the use of an organic extract. Predominantly, however, most studies have investigated DEPs with organic extracts, and thus the findings reported by these studies do not necessarily reflect the specific effects of the particulate content of DEPs. In the cases where organic extractions have been used with the DEP sample (e.g., dichloromethane), a strong extraction of the mutagenic organics (such as specific PAHs) from the particle core can occur. It is a contentious issue, however arguable that this effect would not take place when DEPs are present within the pulmonary microenvironment. Wallace et al. (Wallace et al. 1987), evaluated the effects of DEPs coated with dipalmitoyl lecithin; a surrogate of the lung surfactant fluid; observing a significant mutagenic effect from the TA98 strain of *S. typhimurium*. Wallace et al. (Wallace et al. 1987) speculated that mutagens bound on the particle surface may become solubilised by the surfactant, and that these cause the observed mutagenicity and not the particulate (core) material itself. Contradictory to these findings however, and in line with this study, Belisario and colleagues (Belisario et al. 1984) assessed the mutagenic effects of DEPs in the absence of any organic extracts. Belisario et al. (Belisario et al. 1984) assessed five strains of *S. typhimurium*, including the TA98 strain which elicited the highest mutagenic response, and the TA1535 strain that showed no mutagenicity; supporting the findings of this study. Thus, although it is well reported that DEPs can elicit such effects from *S. typhimurium* neither the precise mechanism nor the interaction between DEPs and bacteria is fully understood.

The finding that none of the nano-objects assessed, particularly the Au-PMA-ATTO and CeO_2 NPs, caused any mutagenic episodes in the TA100 strain supports previous observations by Maenosono et al. (Maenosono et al. 2007) and Kumar et al. (Kumar et al. 2011). Whilst Maenosono et al. (Maenosono et al. 2007) showed FePt NPs; similar to the core-shell technology of the Au-PMA-ATTO NPs used in this study; to cause no significant mutagenicity in the TA98, TA100, TA1535 or TA1537 strains of *S. typhimurium* up to 12.5 $mg.mL^{-1}$, the subsequent conclusions of Kumar et al. (Kumar et al. 2011) were in contrast to this study. In contrast to the TA100 strain, Kumar and colleagues (Kumar et al. 2011) reported significant increases in the number of positive events (bacterial colonies formed) compared to control cultures in the TA98 bacterial strain after TiO_2 and ZnO exposure, contradicting the observation that the metal oxide (CeO_2) NPs used in this study elicited no significant mutagenicity in the TA98 *S. typhimurium* strain.

In addition, the results presented in this study support previous research investigating the effects of CNTs on *S. typhimurium*. Kisin and colleagues (Kisin et al. 2007) reported that SWCNTs elicited no reaction from *S. typhimurium*, whilst Di Sotto et al. (Di Sotto et al. 2009) found no effect in TA98 with three different samples of MWCNTs. Di Sotto and colleagues (Di Sotto et al. 2009) further evaluated the mutagenic effects of different MWCNTs either in the presence or absence of the liver extract S9 mix. They reported that in both environments none of the MWCNTs tested caused any significant ($p > 0.05$) mutagenicity. Wirnitzer et al. (Wirnitzer et al. 2009) also observed no effects with MWCNTs, both with and without S9 mix upon the *S. typhimurium* strains TA98, TA100, TA102, TA1335 and TA1537. Whilst the results of these studies show CNTs to cause minor mutagenic effects to *S. typhimurium*, they also highlight the relevance, or more pertinently the irrelevance of using the S9 mix when testing nano-objects. This extract is used to assess the 'agents' ability to impact upon a specific organ (such as the liver or lung), due to its complex, highly enzymatic environment and to screen for secondary toxic metabolites. Primarily, the lack of positive mutagenic findings in the presence of the S9 mix merely shows that there is no mutagenicity associated with the contaminants present on the surface of CNTs that become DNA-reactive after biotransformation (e.g., PAHs).

Similar to the effects observed with both SWCNTs and MWCNTs, the finding that CAFs, a known pathogen and mutagenic agent (Oberdorster et al. 2007; Donaldson et al. 2010), did not elicit a significant mutagenicity from *S. typhimurium* supports previous research and current perception concerned with asbestos-bacterial interactions. Research by Chamberlain and Tarmy (Chamberlain & Tarmy 1977) and Faux et al. (Faux et al. 1994) reported only insignificant mutagenic effects from *S. typhimurium* following exposure to crocidolite asbestos. Subsequent investigation by Howden and Faux (Howden & Faux 1996) suggested that lipid peroxidation could be the underlying mechanism of action for this material. Thus, similar to DEPs, any effects of crocidolite asbestos observed upon bacterial strains are through indirect means, and not due to a direct interaction between the bacterial DNA and the asbestos fibres. From these studies, a consensus within the particle-fibre toxicology community has been distilled that asbestos is a 'weak mutagen'. Such findings gained from the Ames test for asbestos fibres could suggest an alternative outlook towards the somatic mutation theory (SMT), such as the ability for asbestos fibres to

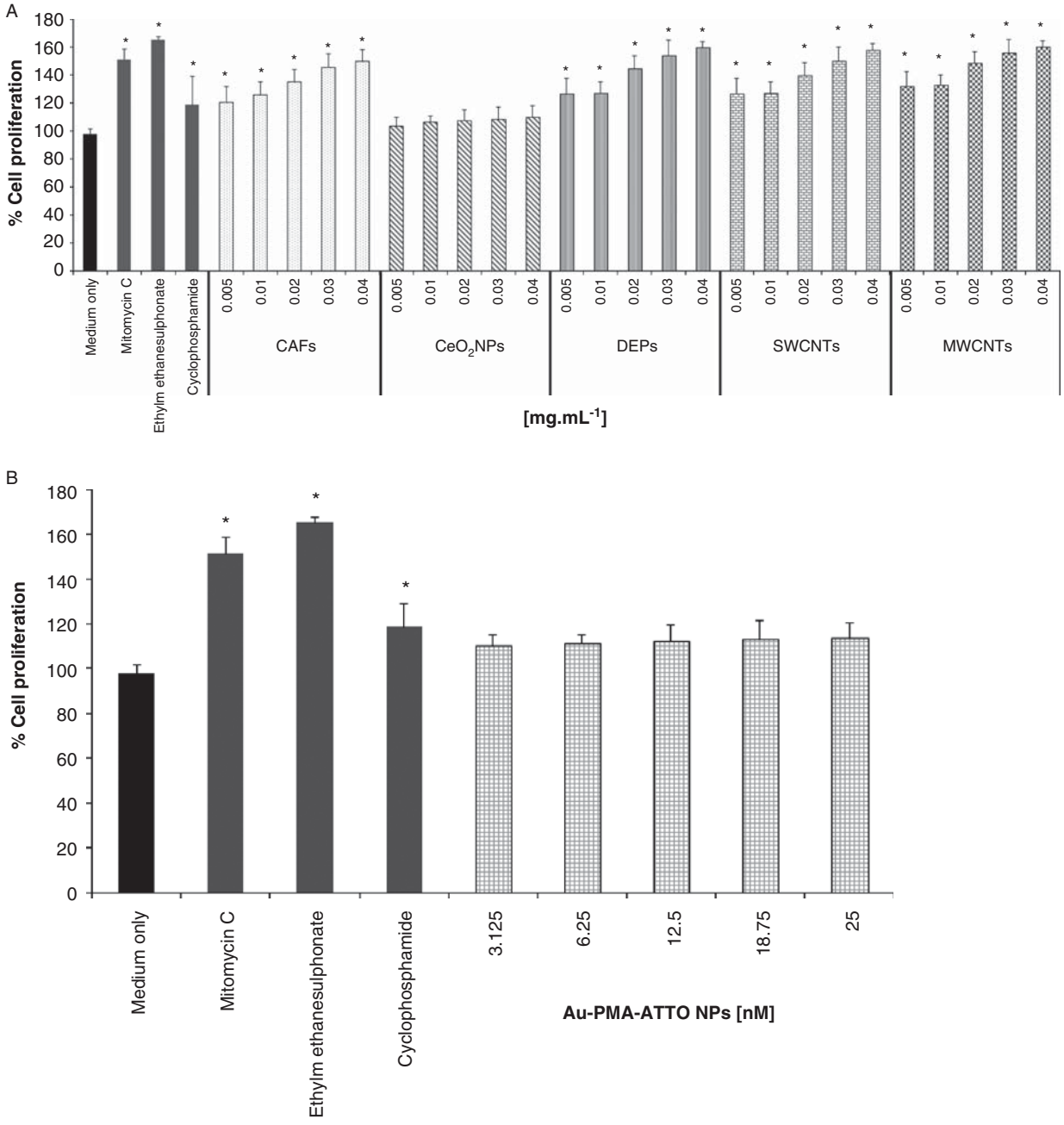

Figure 4. (A) Proliferation status (as determined by the EdU assay) of the 3D *in vitro* triple cell co-culture system of the human epithelial airway barrier after exposure to crocidolite asbestos fibres (CAFs), cerium dioxide (CeO_2) nanoparticles (NPs), diesel exhaust particles (DEPs), single-walled and multi-walled carbon nanotubes (SWCNTs and MWCNTs) at 0.005, 0.01, 0.02, 0.03 and 0.04 mg.mL^{-1} for 48 h at 37°C,5% CO_2. As a positive control, mitomycin C, ethylmethanesulphonate and cyclophosphamide were used at 0.1 mg.mL^{-1}. Data is presented as the mean ± standard error of the mean (SEM). Data has been normalised to a percentage of the baseline (negative control (cell culture media only); taken as 100%). * represents a significant different from baseline of $p < 0.05$. (B) Proliferation status (as determined by the EdU assay) of the 3D *in vitro* triple cell co-culture system of the human epithelial airway barrier after exposure to Au-PMA-ATTO nanoparticles (NPs) at 3.125, 6.25, 12.5, 18.75 and 25 nM for 48 h at 37°C,5% CO_2. As a positive control, mitomycin C, ethylmethanesulphonate and cyclophosphamide were used at 0.1 mg.mL^{-1}. Data is presented as the mean ± standard error of the mean (SEM). Data has been normalised to a percentage of the baseline (negative control (cell culture media only); taken as 100%).

restructure the tissue's architecture (Soto & Sonnenschein 2011). Although the findings of Faux et al. (Faux et al. 1994) as well as Howden and Faux (Howden & Faux 1996) do show that asbestos may elicit a positive response from the Ames test, other, non-mutagenic pathways may be active and should be considered. These include the inactivation of genes by epigenetic alterations, chronic inflammation, cytotoxicity and cell proliferation (Huang et al. 2011).

Because the Ames test is based upon the ability of the *S. typhimurium* to proliferate, and it is important to gain an insight into how the effects of the Ames test relate to the more relevant mammalian cell systems (in respect to the effects of nano-objects towards human health), a comparison was performed with a sophisticated 3D *in vitro* triple cell co-culture model of the human epithelial airway barrier (Rothen-Rutishauser et al. 2008; Blank et al. 2007). Following

exposure of the nano-objects to the triple cell co-culture system for the same period as with the *S. typhimurium*, it was observed that the three fibrous samples used (CAFs, SWCNTs and MWCNTs) caused a significant ($p < 0.05$) concentration-dependent increase in cellular proliferation, indicative of a genotoxic response (Donaldson et al. 2010). These findings are in support of previous literature that has shown that each CAFs, SWCNTs and MWCNTs can cause significant genotoxicity through an increase in cell proliferation *in vitro* (Johnston et al. 2010). The DEPs sample was also found to elicit a significant ($p < 0.05$) genotoxic effect in the form of increased cell proliferation following the 48 h exposure period. It was also observed, however, that the CeO_2 NPs and the Au-PMA-ATTO NPs did not show any form of reduction in cell proliferation, nor, and importantly, any increase in cell proliferation from baseline values (cell culture media only). Whilst the findings for both the CeO_2 NPs and the Au-PMA-ATTO NPs do compare favourable to those eluded from the Ames test analysis, the observations that the highly genotoxic CAFs and mutagenic DEPs, as well as the SWCNTs and MWCNTs cause a different outcome to that shown by the Ames test highlights the inability for this bacterial test to predict or accurately indicate the potential nano-object-associated mutagenicity.

Nano-object interactions with bacteria

The differences observed between the mammalian and bacterial systems as regards nano-based material-associated mutagenicity, together with the discrepancies reported between different bacterial strains and the prominent use of bacterial systems for specific avenues of (eco)nanotoxicological research (Behra & Krug 2008; Kahru & Dubourguier 2010) poses the question, "*how do nano-objects interact with bacteria*?"

Because the TA98 *S. typhimurium* strain is the most commonly used strain for the Ames test (Zhou & Ye 1997; Bunger et al. 1998; Zhou & Ye 1998; Zhao et al. 2004; Singh et al. 2004; Sera et al. 1996; Brayner et al. 2006; Kumar et al. 2011; Maenosono et al. 2007), it was used for all subsequent investigation of the nano-object:bacteria interaction. Interestingly, it was observed that all (nano)fibres (SWCNTs, MWCNTs and CAFs) directly penetrated the membrane of the TA98 strain after a 2 h exposure period at 0.04 $mg.mL^{-1}$. This observation is in contrast to a plethora of previous literature that has reported that whilst asbestos fibres (including CAFs) and MWCNTs interact similarly with biological systems, SWCNTs do not (Johnston et al. 2010). It is also prudent to note, however, that the comparison between asbestos and MWCNTs is only applicable when they share certain physico-chemical characteristics, such as their aspect ratio (length and width) and stiffness (Donaldson et al. 2010). Nonetheless, the finding that all fibre types are able to (i) attach to the bacterial membrane, (ii) penetrate the lipid bilayer or (iii) locate inside Gram negative bacteria shows that the lack of any mutagenic response of these (nano)fibres in the Ames test is independent of their (direct) interaction with the bacterial strain. Similarly, investigation of the CeO_2 NP-bacterial interaction through elemental transmission electron microscopy (Brandenberger et al. 2010) showed these metal oxide NPs to be present on the membrane and inside the TA98 strain. It is important to highlight that none of the nanofibres or the CeO_2 NPs caused any significant ($p > 0.05$) cytotoxicity to the TA98 strain over a 48 h period up to 0.04 $mg.mL^{-1}$. DEPs, however, did elicit a significant ($p < 0.05$) decrease in bacterial viability over a 48 h period, although they were not found to be present inside the bacteria, or interacting with the bacterial membrane. The mutagenic response observed with DEPs can, therefore, be assumed as not associated with a primary interaction between the DEPs and the bacterial DNA, but could be through indirect effects (Schins & Knaapen 2007). These effects could most likely be due to the mutagenic properties of specific components residing in the complex cocktail that is contained within the DEPs, which includes, for example, residue gaseous components (Wilson et al. 2002). Alternatively, the effects observed could be attributed to the DEPs producing free radicals (including reactive oxygen and nitrogen species) which penetrate the bacteria causing a loss in viability and/or mutagenicity. This would not be due to the constituents of the DEPs, but a resultant effect of their heightened ability to cause radical formation and oxidative stress (Oberdorster et al. 2005, 2007). It is important, therefore, that additional research is performed to fully understand these mechanisms in relation to bacteria, as it could be relative to other substances, such as nano-objects engineered from heavy metals (core/shell technologies (i.e., quantum dots with a cadmium core)) (Brandenberger et al. 2010).

In regards to the interesting and surprising observations of the Au-PMA-ATTO NPs, it is not fully understood as to why these NPs interact with the bacteria in this manner, because they are known to interact with eukaryotic cells (i.e., containing a nucleus) with limited adverse effects (Figure 4B) and by a form of endocytosis (Brandenberger et al. 2010; Lehmann et al. 2010). As previously highlighted, and supported by the findings of this study, Maenosono et al. (Mortelmans & Zeiger 2000) reported that FePt NPs did not elicit any mutagenicity to any of the prokaryotic cells (i.e., no nucleus present) used in this study. Maenosono et al. (Maenosono et al. 2007), however, did not go further to understand how these core/shell NPs interacted with these bacterial strains. Because the bacteria used in the Ames test should only replicate (enough to enable colony formation) in the presence of a mutagen, and the Au-PMA-ATTO NPs did not elicit a mutagenic response, it could be suggested that the Au-PMA-ATTO NPs are attracted to the dead bacteria by adhesive forces (Rimai et al. 2000), as well as gaining access to the dead bacteria due to the increase porosity of the membrane. Alternatively, they could be either attracted to the bacteria (i.e., undergoing entry into the bacteria) when they are viable and subsequently elicit a heightened cytotoxic response or enter the bacteria during replication (if the bacteria are able to replicate, if only in a limited capacity). Additional research is therefore necessary to determine the specific interaction of Au-PMA-ATTO NPs and other core/shell NPs with bacteria.

In summary, the results of this study indicate that, independent of the specific physico-chemical characteristics, various different nano-objects are able to directly interact with a form of Gram negative bacteria, either by (i) attaching

to the bacterial membrane, (ii) penetrating the lipid bilayer or (iii) locating inside the bacteria. The direct interaction of Au-PMA-ATTO NPs, CeO_2 NPs, SWCNTs, MWCNTs and CAFs with the *S. typhimurium* did not show a mutagenic effect in the Ames test at the concentrations tested. Comparison against a sophisticated *in vitro* mammalian cell system, however, did show CAFs, SWCNTs and MWCNTs to cause a significant genotoxic effect, in contrast to the Ames test. DEPs were mutagenic in both systems. CeO_2 NPs and Au-PMA-ATTO NPs showed no mutagenic effect in either system. It is important to note, however, that the 'no mutagenic effect' results observed for this panel of nano-objects with the TA98 bacterial strain of *S. typhimurium* is not an indicator for all nano-objects. In particular, when considering the CNTs used in this study, they do not elicit the specific physico-chemical characteristics (i.e., high aspect ratio, or increased stiffness (Donaldson et al. 2010)), or different synthesis methods that have been shown to contribute towards mutagenic effects observed *in vitro* and *in vivo* (Poland et al. 2008; Johnston et al. 2010]. Thus, to fully comprehend the appropriate use of this test in assessing (potential) nano-object mutagenicity, an in-depth panel of nano-objects must be considered.

Conclusions

The findings of this study question the sensitivity of the Ames test for predicting nano-object mutagenicity. Thus, it is suggested that to assess the potential mutagenicity of any nanomaterial within an *in vitro* setting, that a battery of tests are employed, preferably with eukaryotic cells. Due to the novel and complex chemical and physical modes of interaction that nano-objects have with biological systems, assays based upon multicellular systems are particularly promising and relevant, because these provide an essential basis for the investigation of cellular interplay and bystander effects whilst reducing invasive animal testing strategies.

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Declaration of interest

The authors have no conflicts of interest and are entirely responsible for the data contained within and the entire written manuscript.

References

Ames BN, Lee FD, Durston WE. 1973. An improved bacterial test system for the detection and classification of mutagens and carcinogens. Proc Natl Acad Sci USA 70:782–12786.

Aubrecht J, Osowski JJ, Persaud P, Cheung JR, Ackerman J, Lopes SH, et al. 2007. Bioluminescent salmonella reverse mutation assay: a screen for detecting mutagenicity with high throughput attributes. Mutagenesis 22:335–342.

Barnes CA, Elsaesser A, Arkusz J, Smok A, Palus J, Lesniak A, et al. 2008. Reproducible comet assay of amorphous silica nanoparticles detects no genotoxicity. Nano Lett 8:3069–3074.

Behra R, Krug H. 2008. Nanoparticles at large. Nat Nanotechnol 3:253–254.

Belisario MA, Buonocore V, De Marinis E, De Lorenzo F. 1984. Biological availability of mutagenic compounds adsorbed onto diesel exhaust particulate. Mutat Res 135:1–9.

Blank F, Rothen-Rutishauser B, Gehr P. 2007. Dendritic cells and macrophages for a transepithelial network against foreign particulate antigens. Am J Respir Cell Mol Biol 36:669–677.

Bouwmeester H, Lynch I, Marvin HJP, Dawson KA, Berges M, Braguer D, et al. 2011. Minimal analytical characterization of engineered nanomaterials needed for hazard assessment in biological matrices. Nanotoxicology 5:1–11.

Brandenberger C, Clift MJD, Vanhecke D, Muhlfeld C, Stone V, Gehr P, et al. 2010. Intracellular imaging of nanoparticles: Is it an elemental mistake to believe what you see? Part Fibre Toxicol 7:15.

Brandenberger C, Rothen-Rutishauser B, Muhlfeld C, Schmid O, Ferron GA, Maier KL, et al. 2010. Effects of uptake of gold nanoparticles deposited at the air-liquid interface of a human epithelial airway model. Toxicol Appl Pharmacol 242:56–65.

Brayner R, Ferrari-Illiou R, Brivois N, Djediat S, Benedetti MF, Fievet F. 2006. Toxicological impact studies based on Escherichia coli bacteria in ultrafine ZnO nanoparticles colloidal medium. Nano Lett 6:866–870.

British Standards Institution (BSI). 2007. Publicly Available Specification (PAS) 136; Terminology for nanomaterials.

Bunger J, Krahl J, Franke H-U, Munack A, Hallier E. 1998. Mutagenic and cytotoxic effects of exhaust particulate matter of biodiesel compared to fossil diesel fuel. Mutat Res 415:13–23.

Chamberlain M, Tarmy EM. 1977. Asbestos and glass fibres in bacterial mutation tests. Mutat Res 43:159–164.

Claxton LD, Umbuzeiro GdeA, DeMarini DM. 2010. The salmonella mutagenicity assay: the stethoscope of genetic toxicology for the 21st century. Environ Health Perspect 118:1515–1522.

Clift MJD, Gehr P, Rothen-Rutishauser B. 2011. Nanotoxicology: a perspective and discussion of whether or not in vitro testing is a valid alternative. Arch Toxicol 85:723–731.

Collins AR. 2004. The comet assay for DNA damage and repair; principles, applications, and limitations. Mol Biotechnol 26:249–261.

Di Sotto A, Chiaretti M, Carru GA, Bellucci S, Mazzanti G. 2009. Multi-walled carbon nanotubes: Lack of mutagenic activity in the bacterial reverse mutation assay. Toxicol Lett 184:192–197.

Doak SH, Griffiths SM, Manshian B, Singh N, Williams PM, Brown AP, et al. 2009. Confounding experimental considerations in nanogenotoxicology. Mutagenesis 24:285–293.

Donaldson K, Murphy FA, Duffin R, Poland CA. 2010. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. Part Fibre Toxicol 7:5.

Donaldson K, Poland CA, Schins RPF. 2010. Possible genotoxic mechanisms of nanoparticles: Criteria for improved test strategies. Nanotoxicology 4:414–420.

Faux SP, Howden PJ, Levy LS. 1994. Iron-dependent formation of 8-hydroxydeoxyguanosine in isolated DNA and mutagenicity in salmonella typhimurium TA102 induced by crocidolite. Carcinogenesis 15:1749–1751.

Greim H, Norppa H. 2010. Genotoxicity testing of nanomaterials–conclusions. Nanotoxicology 4:421–424.

Grigg J, Tellabati A, Rhead S, Almeida GM, Higgins JA, Bowman KJ, et al. 2009. DNA damage of macrophages at an air-tissue interface induced by metal nanoparticles. Nanotoxicology 3:348–354.

Hakura A, Suzuki S, Satoh T. 1999. Advantage of the use of human liver S9 in the Ames test. Mutat Res 438:29–36.

Hansen SF, Larsen BH, Olsen SI, Baun A. 2007. Categorization framework to aid hazard identification of nanomaterials. Nanotoxicology 1:243–250.

Hartung T. 2011. From alternative methods to a new toxicology. Eur J Pharm Biopharm 77:338–349.
Howden PJ, Faux SP. 1996. Fibre-induced lipid peroxidation leads to DNA adduct formation in Salmonella typhimurium TA104 and rat lung fibroblasts. Carcinogenesis 17:413–419.
Huang SXL, Jaurand M-C, Kamp DW, Whysner J, Hei TK. 2011. Role of mutagenicity in asbestos fiber-induced carcinogenicity and other diseases. J Toxicol Environ Health B Crit Rev 14:179–245.
Iijima S. 1991. Helical microtubules of graphitic carbon. Nature 354:56–58.
International Organization for Standardization (ISO). 2008. Technical Specification (ISO/TS) 27687. 2008. Nanotechnologies – Terminology and definitions for nano-objects – Nanoparticle, nanofibre and nanoplate
Jacobsen NR, Moller P, Cohn CA, Loft S, Vogel U, Wallin H. 2008. Diesel exhaust particles are mutagenic in FE1-Muta™ mouse lung epithelial cells. Mutat Res 641:54–57.
Jacobsen NR, Moller P, Jensen KA, Vogel U, Ladefoged O, Loft S, et al. 2009. Lung inflammation and genotoxicity following pulmonary exposure to nanoparticles in ApoE-/- mice. Part Fibre Toxicol 6:2.
Jacobsen NR, Pojana G, White P, Moller P, Cohn CA, Korsholm KS, et al. 2008. Genotoxicity, cytotoxicity, and reactive oxygen species induced by single-walled carbon nanotubes and C60 fullerenes in the FE1-Muta™ mouse lung epithelial cells. Environ Mol Mutagen 49:476–487.
Jaurand MC, Renier A, Daubriac J. 2009. Mesothelioma: do asbestos and carbon nanotubes pose the same health risk? Part Fibre Toxicol 12:16.
Johnston HJ, Hutchison GR, Christensen FM, Peters S, Hankin S, Aschberger K, et al. 2010. A critical review of the biological mechanisms underlying the in vivo and in vitro toxicity of carbon nanotubes: the contribution of physico-chemical characteristics. Nanotoxicology 4:207–246.
Kahru A, Dubourguier H-C. 2010. From ecotoxicology to nanecotoxicology. Toxicology 269:105–119.
Kirsch-Volders M, Plas G, Elhajoujji A, Lukamowicz M, Gonzalez L, Loock KV, et al. 2011. The in vitro MN assay in 2011: origin and fate, biological significance, protocols, high throughput methodologies and toxicological relevance. Arch Toxicol 85:873–899.
Kisin ER, Murray AR, Keane MJ, Shi XC, Schwegler-Berry D, Gorelik O, et al. 2007. Single-walled carbon nanotubes: geno- and cytotoxic effects in lung fibroblast V79 cells. J Toxicol Environ Health A 70:2071–2079.
Krug H, Wick P. 2011. Nanotoxicology: an interdisciplinary challenge. Angew Chem Int Ed Engl 50:1260–1278.
Kuhlbusch TAJ, Asbach C, Fissan H, Gohler D, Stintz M. 2011. Nanoparticle exposure at nanotechnology workplaces: a review. Part Fibre Toxicol 8:22.
Kumar A, Pandey AK, Singh SS, Shanker R, Dhawan A. 2011. Cellular uptake and mutagenic potential of metal oxide nanoparticles in bacterial cells. Chemosphere 83:1124–1132.
Landsiedel R, Kapp MD, Schulz M, Wiench K, Oesch F. 2009. Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations—Many questions, some answers. Mutat Res 681:241–258.
Landsiedel R, Ma-Hock L, Van Ravenzwaay B, Schulz M, Wiench K, Champ S, et al. 2010. Gene toxicity studies on titanium dioxide and zinc oxide nanomaterials used for UV-protection in cosmetic formulations. Nanotoxicology 4:364–381.
Lehmann AD, Parak WJ, Zhang F, Ali Z, Rocker C, Nienhaus GU, et al. 2010. Fluorescent-magnetic hybrid nanoparticles induce a dose-dependent increase in proinflamatory response in lung cells in vitro correlated with intracellular localization. Small 6:753–762.
Lin C-AJ, Sperling RA, Li JK, Yang T-Y, Li P-Y, Zanella M, et al. 2008. Design of an amphiphilic polymer for nanoparticle coating and functionalization. Small 4:334–341.
Lindberg JK, Falck GC-M, Suhonen S, Vippola M, Vanhala E, Catalan J, et al. 2009. Genotoxicity of nanomaterials: DNA damage and micronuclei induced by carbon nanotubes and graphite nanofibres in human bronchial epithelial cells in vitro. Toxicol Lett 186:166–173.
Liu S, Wei L, Hao L, Fang N, Chang MW, Xu R, et al. 2009. Sharper and faster "nano darts" kill more bacteria: A study of antibacterial activity of individually dispersed pristine single-walled carbon nanotube. ACS Nano 3:3891–3902.
Madigan MT, Martinko JM, Dunlap PV, Clark DP. 2008. Brock biology of microorganisms. 12th edition. San Francisco, CA, USA: Benjamin Cummings.
Maenosono S, Suzuki T, Saita S. 2007. Mutagenicity of water-soluble FePt nanoparticles in Ames test. J Toxicol Sci 32:575–579.
Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, Oberdorster G, et al. 2006. Safe handling of nanotechnology. Nature 444:267–269.
Maynard AD. 2007. Nanotechnology: the next big thing, or much ado about nothing? Ann Occup Hyg 51:1–12.
Morris J, Willis J, De Martinis D, Hansen B, Laursen H, Sintes JR, et al. 2011. Science policy considerations for responsible nanotechnology decisions. Nat Nanotechnol 6:73–77.
Mortelmans K, Zeiger E. 2000. The ames salmonella/microsome mutagenicity assay. Mutat Res 455:29–60.
Ng CT, Li JJ, Bay BH, Yung LY. 2010. Current studies into the genotoxic effects of nanomaterials. J Nucleic Acids 2010:947859.
Oberdorster G, Oberdorster E, Oberdorster J. 2005. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 113:823–839.
Oberdorster G, Stone V, Donaldson K. 2007. Toxicology of nanoparticles: a historical perspective. Nanotoxicology 1:2–25.
Oberdorster G. 2010. Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology. J Intern Med 267:89–105.
Organisation for Economic Co-operation and Development (OECD). Technical Guide for Chemical Testing 471; Adopted 1997.
Organisation for Economic Co-operation and Development (OECD). 2011. Working Party on Nanotechnology (WPN). www.oecd.org. Date accessed 21 October 2011.
Pan X, Redding JE, Wiley PA, Wen L, McConnell JS, Zhang B. 2010. Mutagenicity evaluation of metal oxide nanoparticles by the bacterial reverse mutation assay. Chemosphere 79:113–116.
Pfaller T, Colognato R, Nelissen I, Favilli F, Casals E, Ooms D, et al. 2010. The suitability of different cellular in vitro immunotoxicity and genotoxicity methods for the analysis of nanoparticle-induced events. Nanotoxicology 4:52–72.
Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WAH, Seaton A, et al. 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogencity in a pilot study. Nat Nanotechnol 3:423–428.
Raemy DO, Limbach LK, Rothen-Rutishauser B, Grass RN, Gehr P, Birbaum K, et al. 2011. Cerium oxide nanoparticle uptake kinetics from the gas-phase into lung cells in vitro is transport limited. Eur J Pharm Biopharm 77:368–375.
Rimai DS, Quesnel DJ, Busnaia AA. 2000. The adhesion of dry particles in the nanometer to micrometer size range. Colloids Surf A Physicochem Eng Asp 165:3–10.
Roller M. 2009. Carcinogenicity of inhaled nanoparticles. Inhal Toxicol 21:144–157.
Rothen-Rutishauser B, Blank F, Muehlfeld C, Gehr P. 2008. In vitro models of the human epithelial airway barrier to study the toxic potential of particulate matter. Expert Opin Drug Metab Toxicol 4:1075–1089.
Ryman-Rasmussen JP, Cesta MF, Brody AR, Shipley-Phillips JK, Everitt JI, Tewksbury EW, et al. 2009. Inhaled carbon nanotubes reach the subpleural tissue in mice. Nat Nanotechnol 4:747–751.
Schins RPF, Knaapen AM. 2007. Genotoxicity of poorly soluble particles. Inhal Toxicol 19:189–198.
Sera N, Tokiwa H, Miyata N. 1996. Mutagenicity of the fullerene C60-generated singlet oxygen dependent formation of lipid peroxides. Carcinogenesis 17:2163–2169.
Singh N, Manshian B, Jenkins GJS, Griffiths SM, Williams PM, Maffeis TGG, et al. 2009. Nanogenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30:3891–3914.
Singh P, DeMarini DM, Dick CAJ, Tabor DG, Ryan JV, Linak WP, et al. 2004. Sample characterization of automobile and forklift diesel exhaust particles and comparative pulmonary toxicity in mice. Environ Health Perspect 112:820–825.
Soto AM, Sonnenschein C. 2011. The tissue organization field theory of cancer: a testable replacement for the somatic mutation theory. Bioessays 33:332–340.
Stone V, Johnston H, Schins RPF. 2009. Development of in vitro systems for nanotoxicology: methodological considerations. Crit Rev Toxicol 39:613–626.
Takagi A, Hirose A, Nishimura T, Fukumori N, Ogata A, Ohashi N, et al. 2008. Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotube. J Toxicol Sci 33:105–116.
Teeguarden JG, Hinderliter PM, Orr G, Thrall BD, Pounds JG. 2007. Particokinetics in vitro: dosimetry consideration for in vitro nanoparticle toxicity assessements. Toxicol Sci 95:300–312.
Thurnherr T, Su DS, Diener L, Weinberg G, Manser P, Pfaender N, et al. 2009. Comprehensive evaluation of in vitro toxicity of three

large-scale produced carbon nanotubes on human Jurkat T cells and a comparison to crocidolite asbestos. Nanotoxicology 3:319–338.
Wallace WE, Keane MJ, Hill CA, Xu J, Ong TM. 1987. Mutagenicity of diesel exhaust particles and oil shale particles dispersed in lecithin surfactant. J Toxicol Environ Health 21:163–171.
Warheit DB, Donner EM. 2010. Rationale of genotoxicity testing of nanomaterials: regulatory requirements and appropriateness of aavailable OECD test guidelines. Nanotoxicology 4:409–413.
Wick P, Clift MJD, Rosslein M, Rothen-Rutishauser B. 2011. A brief summary of carbon nanotubes science and technology: A health and safety perspective. ChemSusChem 4:905–911.
Wick P, Manser P, Limbach LK, Dettlaff-Weglikowska U, Krumreich F, Roht S, et al. 2007. The degree and kind of agglomeration affect carbon nanotube cytotoxicity. Toxicol Lett 168:121–131.
Wilson MR, Lightbody JH, Donaldson K, Sales J, Stone V. 2002. Interactions between ultrafine particles and transition metals in vivo and in vitro. Toxicol Appl Pharmacol 184:172–179.
Wirnitzer U, Herbold B, Voetz M, Ragot J. 2009. Studies on the in vitro genotoxicity of baytubes, agglomerates of engineered multi-walled carbon-nanotubes (MWCNT). Toxicol Lett 186:160–165.
Zhao HW, Barger MW, Ma JKH, Castranova V, Ma JYC. 2004. Effects of exposure to diesel exhaust particles (DEP) on pulmonary metabolic activation of mutagenic agents. Mutat Res 564:103–113.
Zhou W, Ye SH. 1997. Mutagenicity of scooter exhaust particulate matter. J Toxicol Environ Health Part A 52:35–44.
Zhou W, Ye S-H. 1998. Effects of two new lubricants on the mutagenicity of scooter exhaust particulate matter. Mutat Res 414:131–137.

Supplementary material available online

Supplementary Table I.