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Review

NanoGenotoxicology: The DNA damaging potential of engineered nanomaterials

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ABSTRACT

With the rapid expansion in the nanotechnology industry, it is essential that the safety of engineered nanomaterials and the factors that influence their associated hazards are understood. A vital area governing regulatory health risk assessment is genotoxicology (the study of genetic aberrations following exposure to test agents), as DNA damage may initiate and promote carcinogenesis, or impact fertility. Of late, considerable attention has been given to the toxicity of engineered nanomaterials, but the importance of their genotoxic potential on human health has been largely overlooked. This comprehensive review focuses on the reported abilities of metal nanoparticles, metal-oxide nanoparticles, quantum dots, fullerenes, and fibrous nanomaterials, to damage or interact with DNA, and their ecogenotoxicity is also considered. Many of the engineered nanomaterials assessed were found to cause genotoxic responses, such as chromosomal fragmentation, DNA strand breakages, point mutations, oxidative DNA adducts and alterations in gene expression profiles. However, there are clear inconsistencies in the literature and it is difficult to draw conclusions on the physico-chemical features of nanomaterials that promote genotoxicity, largely due to study design. Hence, areas that require that further attention are highlighted and recommendations to improve our understanding of the genotoxic potential of engineered nanomaterials are addressed.

1. Introduction

The nanotechnology industry is rapidly growing with promises of substantial benefits that will have significant economic and scientific impacts, applicable to a whole host of areas ranging from aerospace engineering and nano-electronics to environmental remediation and medical healthcare. The design and development of novel engineered nanomaterials have been of fundamental importance to the industry due to the beneficial physico-chemical features they offer. Some of which include improved thermal and/or electrical conductivity, substantially harder and stronger materials (yet lighter than metals such as steel), improved catalytic activity and advanced optical properties. Currently, estimates indicate that there are over 800 consumer products already available containing nanomaterials (Fig. 1; Woodrow Wilson Database), the sales of which were valued at $147 billion in 2007 and are expected to soar over the coming years with a predicted value of $3.1 trillion by 2015 [1]. Hence, human exposure is already occurring and is set to increase dramatically in the coming years.

A nanomaterial is defined as a substance with at least one dimension <100 nm in size and they can take many different forms such as tubes, rods, wires or spheres, with more elaborate structures devised, such as nano-onions and nanopeapods [2,3]. However, their small size in addition to the novel physico-chemical properties may also be responsible for adverse biological effects. Consequently, over the last 4 yrs the health and environmental safety of nanomaterials has drawn increasing attention, with the first report published in 2004 by the Royal Society and Royal Academy of Engineering [4] highlighting the distinct lack of information on human health and environmental impacts of engineered nanomaterials. Several other governmental reports have emerged since [5–7], but in this time, the safety considerations have certainly not paralleled the growth of the material development side of the nanotechnology industry. Furthermore, the limited information that is slowly emerging does indicate that these substances induce cytotoxicity, oxidative stress and inflammatory responses [8–11]. Thus given the considerable uncertainty about the safety of engineered nanomaterials, it is imperative that we understand and thereafter minimise any potential toxicological hazards associated with them, not only to protect human health and the environment, but also to avoid damaging the nanotechnology industry in the longer term.

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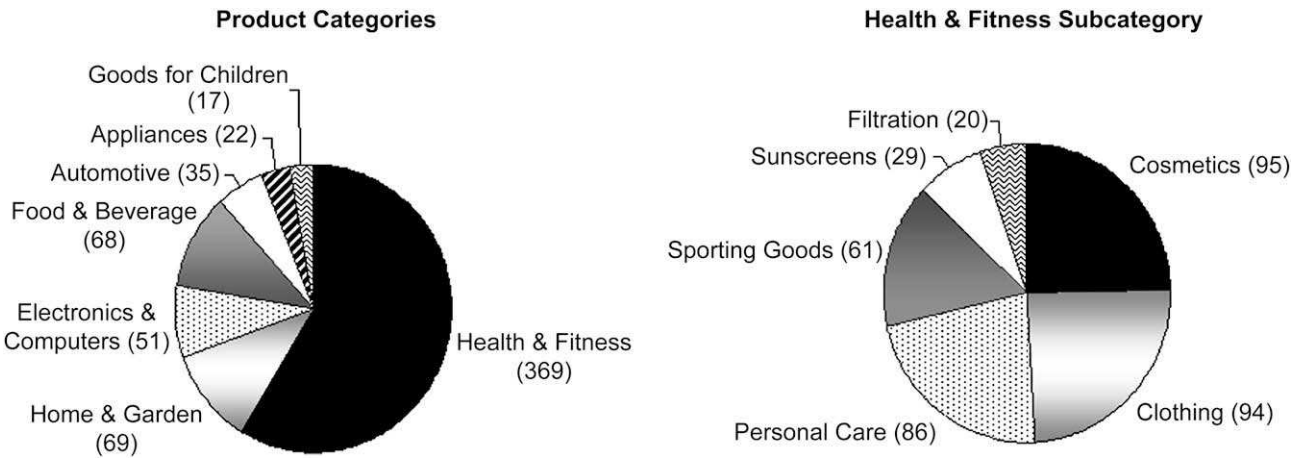

Fig. 1. Consumer products currently available that contain nanomaterials. Adapted from the Woodrow Wilson Database in Jan 2009 (http://www.nanotechproject.org).

Over the last 5 yrs the number of publications focusing on nanotoxicology has slowly gained momentum, but the majority of these studies have primarily assessed cytotoxicity, thus in many cases very high exposure doses have been required. However, these investigations overlook the subtle cellular alterations that may arise at lower concentrations, which may not result in cell death but could contribute to human health risks. The most important example here is DNA damage induction, due to the fact that classic genotoxins (that cause genetic alterations in the absence of cell death) lead to carcinogenesis. Hence, a key area governing health risk assessment of new pharmaceuticals and chemicals is genotoxicology, involving the study of genetic damage following exposure to test substances. Such information is vital as DNA damage could not only initiate cancer development, but can also have an impact upon fertility and the health of subsequent generations if disturbances arise in reproductive cells. Consequently, genotoxicity testing and thus the evaluation of the carcinogenic or mutagenic potential of new substances is an important part of preclinical safety testing of novel pharmaceuticals, which is a necessity before embarking on Phase I/II clinical trials. Additionally, it is a central element of risk assessment for any chemical compound that humans may be routinely exposed to (for example in foods or personal care products). However, despite its importance to human health, the genotoxicity of nanomaterials has so far largely been overlooked.

Due to their small size and high surface area, coupled to other physico-chemical features such as metal contaminants and charged surfaces, nanomaterials may well have unpredictable genotoxic properties. They may cause DNA damage indirectly, by promoting oxidative stress and inflammatory responses. Alternatively, if small enough, they may pass through cellular membranes and gain access to the nucleus where they may interact directly with DNA, causing damage. Additionally, if nanomaterials were able to accumulate within a cell but not necessarily gain access to the nucleus, they may still come into direct contact with DNA during mitosis when the nuclear membrane breaks down, providing ample opportunity for DNA aberrations to arise.

With regards to many nano-based products, such as those designed to improve computer chip technology, automotive catalytic converters or thermal barrier coatings for the aerospace industry, occupational exposure of the workforces in these industries may be of particular concern, but it is unlikely that the general public would be exposed to the raw nanomaterials in high quantities. However, nanomaterials are already being utilised in several cosmetics (lipstick, sunscreen, anti-aging creams), while current medical sources of exposure include dental prosthesis and orthopaedic implant wear debris [12–14]. Additionally, in the future there is promise that nanomaterials could be administered as diagnostic aids, drug carriers or therapeutic treatments for patients [15,16]. Thus, with current and near-future exposure scenarios, it appears that workforces in the nanotechnology industries are likely to have the highest levels of chronic exposure to nanomaterials; patients receiving nanomaterial-based treatments would have high-to-medium exposures but over limited periods of time; while consumers would likely have low, chronic exposures. Given the wide-ranging personal exposure scenarios, and the fact that with clinical applications, individuals that might be more vulnerable due to their pre-existing medical conditions, may be exposed to nanomaterials, it is therefore crucial that their genotoxic potential and underlying mechanisms of action are established.

Furthermore, there is a distinct need to identify associations between genotoxic responses and specific nanomaterial physico-chemical features, because if such good quality data is available for some nanomaterials, clear (geno)toxicological trends may begin to emerge. Thus, in the future it may be possible to extrapolate risks to other nanomaterials with similar characteristics and this information may also prove crucial in enabling the nanotechnology industry to mould their design and fabrication of new nanomaterials to ensure their biocompatibility. This review will therefore focus on the genotoxicity of nanomaterials reported to date (summarised in Table 1) and will attempt to shed light on the possible mechanisms involved in their promotion of DNA damage following exposure. The description of both specific terminology and commonly used experimental systems utilised in genotoxicology is provided in Boxes 1 and 2 respectively, to assist the reader throughout this publication. Additionally, areas of work that require further attention and recommendations to improve our understanding of the genotoxic potential of engineered nanomaterials will be addressed.

2. Mechanisms of nanomaterial induced DNA damage

If nanomaterials are able to gain entry into the body via inhalation, dermal or oral routes there are a number of direct and indirect mechanisms that can subsequently promote DNA damage. Nanomaterials may be able to penetrate into the cell through a number of mechanisms (Fig. 2) and subsequently the nucleus, either through diffusion across the nuclear membrane (if they are small enough), transport through the nuclear pore complexes, or they may become enclosed in the nucleus by chance following mitosis as the nuclear membrane dissolves during cell division and then reforms in each daughter cell. If the nanomaterials were to locate within the nucleus, then direct interaction between them

Table 1
Summary of nanomaterial gentoxicity studies to date.

Citation	Nanoparticle tested [size]	*In vitro*/*in vivo*	Genotoxicity assays	Toxicity assays	Other analysis	Physico-chemical characterisation	Findings
Gold nanoparticles							
Li et al., 2008 [47]	Au NP (0.5–1 nM) [20 nm]	Human fetal lung fibroblast cell line (MRC-5)	HPLC to measure 8-OHdG	Cell proliferation (trypan blue)	Gene expression profiling	None	- Significant oxidative DNA damage. - No increase in cell death. - DNA repair genes down-regulated (cyclin C, Hus1, BRCAI/BRCC1).
Silver nanoparticles							
Ahamed et al., 2008 [52]	Ag NP [25 nm]	Mouse embryonic stem cells (MES) and fibroblasts (MEF)	Expression of DNA repair proteins; H2AX phosphorylation	MTT assay	p53 Phosphorylation	None	- Increased expression of Rad51, p53 and phospho- H2AX proteins. - Time-dependent decrease in cell viability.
Cha et al., 2008 [49]	Ag NP [13 nm]	7-Week-old male balb/c mice. Human liver cell line (Huh-7)		MTT assay	Glutathione production; DNA content; gene expression profiling	TEM	- No cytotoxicity or change in glutathione production. - Altered expression patterns of genes involved in apoptosis and inflammation.
Cobalt and cobalt–chromium nanoparticles							
Colognato et al., 2008 [56]	Co NP [100–500 nm, median 256 nm]	Human peripheral blood leukocytes	Micronucleus (Mn) assay; Comet assay	Cytokinesis-block proliferation index (CBPI)	None	None	- Dose-dependent increase in DNA strand breakages and micronucleus (Mn) frequency at sub-cytotoxic doses.
Papageorgiou et al., 2007 [27]	Co–Cr alloy [30 nm]	Primary human dermal fibroblast	Mn assay; Comet assay; 8-OHdG detection	MTT and LDH assays	Electron paramagnetic resonance; cytokine analysis	None	- Significant generation of superoxide and hydroxyl radicals. - Dose-dependent increase in Mn frequency, % comet tail and cytotoxicity.
Davies et al., 2005 [64]	Synovial fluid from patients with failed Co–Cr hip implants	Primary human fibroblasts	Comet assay			Electrothermal atomic absorption spectroscopy to analyse metal content	- Co–Cr synovial fluid caused DNA damage, but synovial fluids from failed stainless-steel metal-on-polyethylene implants did not.
Daley et al., 2004 [62]	Peri-prosthetic tissue from patients with Co–Cr alloy hip implants	Peripheral blood lymphocytes (PBL); primary amnion cells	Chromosome 1, 2 and 3 painting (PBL); Mn assay (amnion cells)			Tissue metal content: double focusing, magnetic sector inductively coupled plasma mass spectrometer	- Dose-dependent increase in Mn with isolated wear debris, but no difference between Co–Cr and titanium alloy samples. - PBL from patients demonstrated aneuploidy, which correlated with titanium concentration.
Ladon et al., 2004 [63]	Patients with Co–Cr alloy hip implants	PBL	Chromosome 1, 2 and 3 painting			Whole blood metal content: high-resolution inductively coupled plasma mass spectrometry	- Patients demonstrated increased Co levels, Cr levels, aneuploidy and chromosomal translocations 2 yrs after joint arthroplasty. - No correlation observed between DNA damage and Co or Cr content.
Titanium dioxide nanoparticles							
Gopalan et al., 2009 [189]	Anatase TiO_2 and ZnO [both were 40–70 nm]	PBL; human sperm cells	Comet assay in dark (D), under pre-irradiation (PI) and simultaneous irradiation (SI) conditions.	Trypan blue staining		SEM, photon correlation spectroscopy (Zetasizer)	- Dose-dependent increase in DNA damage with TiO_2 and ZnO, in both cell types. - Sperm did not show a photogenotoxic response, but lymphocytes did (i.e. damage was in the order $SI \geq PI > D$).

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Table 1 (*continued*)

Citation	Nanoparticle tested [size]	*In vitro*/*in vivo*	Genotoxicity assays	Toxicity assays	Other analysis	Physico-chemical characterisation	Findings
Xu et al., 2009 [76]	Anatase TiO_2 [5 and 40 nm] and C_{60} fullerenes	*gpt* Delta transgenic MEF cells	*gpt* Delta transgenic mutation assay	MTT assay	Fluorescence detection of peroxynitrite anions ($ONOO^-$)	BET surface area analysis	- Dose-dependent induction of $ONOO^-$ with TiO_2 and C_{60} - TiO_2 5 nm: mutagenic >0.1 μg/ml, but no dose dependency. - TiO_2 40 nm: dose-dependent increase in mutagenicity (>0.1 μg/ml) - C_{60}: only mutagenic at 10 μg/ml - Endocytosis, NOS and COX-2 inhibitors suppress mutagenicity in all cases.
Kang et al., 2008 [46]	TiO_2 [25 nm]	PBL	Mn assay; Comet assay	Trypan blue staining	ROS measurements; p53 phosphorylation	None	- Dose and time-dependent decrease in cell viability. - Dose-dependent increase in Mn frequency and ROS generation. - Time-dependent up-regulation of phospho- and total-p53.
Karlsson et al., 2008 [26]	CuO, TiO_2, ZnO, $CuZnFe_2O_4$, Fe_2O_3, Fe_3O_4, carbon nanotubes	A549 type II lung epithelial cells	Comet assay	Trypan blue staining	ROS measurements (DCFDA); oxidative DNA lesions	TEM, DLS, Zeta potential	- Dose-dependent increase in DNA damage induced by CuO > TiO_2 > $CuZnFe_2O_4$ > carbon nanotubes. - Fe_2O_3, Fe_3O_4 and carbon did not significantly increase DNA damage. - Oxidative lesions caused by CuO, ZnO, $CuZnFe_2O_4$ and Fe_3O_4. - CuO induced intracellular ROS.
Park et al., 2008 [29]	TiO_2 [21 nm]	Human bronchial epithelial cell line (BEAS-2B)		MTT assay	Measurement of ROS and GSH; caspase-3 activity; chromosome condensation; gene expression analysis.	None	- Cell viability decreased in a time- and dose-dependent manner. - Dose-dependent increase in chromosome condensation, caspase-3 and ROS generation; decrease in GSH. - Induced expression of oxidative stress and inflammation related genes.
Theogaraj et al., 2007 [77]	Ultrafine TiO_2 with organic and inorganic coatings [14–60 nm]	Chinese hamster ovary cells (CHO)	Chromosome aberration test			TEM, X-ray diffraction, X-ray disc centrifugation	- No increase in chromosomal damage frequencies observed in the presence or absence of UV.
Warheit et al., 2007 [188]	Rutile TiO_2 coated with alumina (AT) [136 nm], or silica and alumina (AST) [149 nm]; 80% anatase/20% rutile TiO_2 (ART) [129 nm]	*Salmonella typhimurium* (TA98, TA100, TA1535, TA1537), *E. coli* (WP2*uvr*A); CHO cells	Ames test +/− S9; chromosome aberration test	*In vivo* oral and pulmonary toxicity in rats; acute dermal and ocular irritation in rabbits; dermal sensitisation in mice	Toxicity in aquatic species (Rainbow trout, daphnids, green algae)	X-ray diffraction, X-ray fluorescence, DLS, BET surface area analysis	- Ames test negative up to 5000 μg/plate. - No chromosome abberations induced up to 2500 μg/ml (4 h, −S9), 250 μg/ml (4 h, +S9), or 100 μg/ml (20 h, −S9). - Ranking of pulmonary toxicity: ART > AT = AST. - Limited toxicity in aquatic species.
Zhu et al., 2007 [210]	TiO_2 [10 nm]	Naked plasmid DNA	Plasmid nicking assay			None	- No binding to naked plasmid DNA evident. Reports of binding to calf thymus DNA.
Gurr et al., 2005 [28]	TiO_2 [10 and 20 nm]	BEAS-2B cells	Comet assay; Mn assay	MTT assay	Lipid peroxidation; ROS measurements	Sizing of nanoparticles	- 10, 20, 200 nm (anatase) and 200 nm (rutile) particles induce oxidative DNA damage. - Only 10 and 20 nm anatase cause Mn. - TiO_2 induced H_2O_2 and NO.

Rahman et al., 2002 [75]	Ultrafine TiO_2 [≤20 nm]	Syrian hamster embryo cells (SHE)	Mn assay with kinetochore staining.	Trypan blue staining	DNA fragmentation as apoptosis measure	None	- Significant increase in Mn between 0.5 and 5 μg/cm^2. - Increased cytotoxicity at higher doses (>10 μg/cm^2).
Zinc oxide nanoparticles							
Dufour et al., 2006 [78]	Zinc oxide [mean particle size 100 nm]	CHO cells	Chromosomal aberration test in dark (D), under pre-irradiation (PI) and simultaneous irradiation (SI) conditions.	Cytotoxicity measured as % decrease in population doublings		None	- The cytotoxicity was as follows: SI > PI > D. - At ZnO concentration with similar cytotoxicity, chromosome aberrations were nearly identical for SI and PI. - ZnO was found to be clastogenic under all conditions: SI > PI > D.
Silica nanoparticles							
Barnes et al., 2008 [86]	SiO_2 [30, 80, 400 nm]	3T3-L1 fibroblasts	Comet assay	MTT assay; trypan blue staining.		TEM, DLS, Zeta potential	- No significant genotoxicity observed at any concentration or time-point (3, 6 or 24 h)
Jin et al., 2007 [85]	SiO_2 doped with luminescent dyes (RuBpy and TMR) [50 nm]	A549 cells	Comet assay; 8-OHdG adduct measurement	MTT assay; trypan blue staining.	Repair activity (using oligos containing 8-OHdG)	None	- No comets, even at 0.5 mg/mL, - No 8-OHdG adducts, or repair activity, but OGG1 up-regulated. - Toxicity evident at 0.1 and 0.5 mg/mL
Wang et al., 2007 [87]	SiO_2 [6.57, 8.2, 196.52 nm]	WIL2-NS human lymphoblastoid cells	Mn assay; Comet assay; HPRT assay	MTT assay; trypan blue staining.		High performance particle sizer	- Cytotxicity at doses >120 μg/mL. - Mn induced at 30–120 μg/mL. - No increases in comet tail. - Significant increase in HPRT mutations at 120 μg/mL.
Iron oxide nanoparticles							
Auffan et al., 2006 [101]	DMSA-coated Maghemite nanoparticles (nano-γFe_2O_3) [<70 nm]	Normal human fibroblasts	Comet assay	Cytotoxicity assay (WST-1)		TEM, X-ray diffraction, BET surface area analysis, DLS, X-ray absorption spectroscopy	- No genotoxicity observed. - Cell viability decreased from 10^{-6} to 10^{-3} g/L.
Sadeghiani et al., 2005 [92]	Polyaspartic acid-coated magnetite nanoparticles [8.5 nm]	Female swiss mice	Mn assay	Cytotoxicity assay (% PCEs)		TEM	- Time- and dose-dependent Mn increases. - No change in blood cell population.
Quantum dots							
Anas et al., 2008 [108]	CdSe capped with ZnS shell and streptavidin functionalised	Naked plasmid DNA	Plasmid nicking assay			None	- Photoactivated QDs cause strand breakages and nucleobase damage in plasmid DNA.
Choi et al., 2008 [45]	CdTe	Human breast carcinoma cells		MTT assay	Histone modifications	None	- Chromatin condensation, nuclear content reorganisation and loss of mitochondrial cristae observed. - Global hypoacetylation of histone-3 and activation of p53.
Green and Howman 2004 [107]	CdSe capped with a ZnS shell and biotin functionalised.	Naked plasmid DNA	Plasmid nicking assay		Reactive oxygen intermediates measured	None	- Induced 56% and 29% damage in the presence and absence of UV respectively; attributed to free radicals (surface oxide- and photo-generated).
Hoshino et al., 2004 [105]	QD-COOH [18.03 ± 6.76 nm]	WTK1 cells	Comet assay	MTT		DLS	- Decreased cell proliferation 0.5–2 μM. - Increase in comet tail length after 2 h, but did not persist at 12 h (possibly efficient DNA repair).

(*continued on next page*)

Table 1 (*continued*)

Citation	Nanoparticle tested [size]	*In vitro*/*in vivo*	Genotoxicity assays	Toxicity assays	Other analysis	Physico-chemical characterisation	Findings
Fullerenes							
Mori et al., 2006 [120]	Mixture of C_{60} and C_{70}, fullerite	Sprague–Dawley rats; *Salmonella typhimurium* (TA100, TA1535, TA98, TA1537), *E. coli* (WP2*uvrA*/pKM101); Chinese hamster lung cells (CHL)	Ames test; chromosome aberration test	Single dose oral toxicity study		None	- No toxicity observed. - No mutagenicity detected by Ames test up to 5000 μg/plate. - No chromosomal aberrations detected up to 5000 μg/mL.
Dhawan et al., 2006 [115]	Aqu/nC_{60} and EthOH/nC_{60} suspensions	PBL	Comet assay			TEM	- Aqu/nC_{60} suspensions elicited higher genotoxic response than EthOH/nC_{60} at the same dose.
Yasuharu and Naoharu, 2006 [118]	Water soluble C_{60} fullerenes $C_{60}(OH)_{24}$ [7.1 ± 2.4 nm]	CHO cells; HeLa cells; human embryonic kidney cells (HEK293)	Mn assay	Cell proliferation analysis, LDH activity		None	- After 33 days HEK293 and HeLa cells showed increased cell proliferation, but not LDH activity. - All cells showed increased genotoxicity (Mn frequency).
Babynin et al., 2002 [119]	Three novel C_{60} fullerene derivatives	*Salmonella typhimurium* (BA13 strain)	Ames test			None	- Antimutagenic effect seen with [61] dimethoxyphosphoryl [61] carbethoxy-methanofullerene [60] exposed to visible light. - No differences observed between control and [61] dimethoxyphosphoryl [61] carbethoxy-methano [60] fullerene, or 1-methyl-2-(3,5-ditretbutyl-4-hydroxy-phenyl)3,4-fulleropyrrolidine).
Takenaka et al., 1999 [117]	Water soluble cationic fullerene derivative	Calf thymus DNA	Cyclic voltammetry			None	- Isomeric fulleropyrrolidine readily dissolved in water and bound to DNA forming a DNA–fullerene complex
Sera et al., 1996 [116]	C_{60}-fullerenes	*Salmonella typhimurium* (TA102, TA104, YG3003 a repair enzyme-deficient mutant of TA102)	Ames test		ESR spectrum analysis	None	- Mutagenicity elevated in YG3003. - 8-OHdG adducts formed. - ROS generated by irradiating the C_{60} by visible light. - Antioxidants β-carotene and para-bromo-phenacyl bromide reduced mutation frequency.
Carbon nanotubes							
Jacobsen et al., 2008 [111]	- C_{60} 99.9% pure [0.7 nm] - SWCNT [0.9–1.7 nm diameter, <1 μm length] - Carbon Black (CB) [14 nm]	FE1Muta Mouse lung epithelial cell line	Comet assay; *cII* mutation analysis	LDH activity		TEM, BET surface area analysis, DLS	- SWCNT and C_{60} are less genotoxic than CB and diesel exhaust particles. - CB and SWCNT caused a dose-dependent increase in ROS. - With C_{60} ROS production was lower.
Muller et al., 2008 [145]	MWCNT, 98% purity [11.3 nm diameter, 700 nm length]	Type II pneumocytes (AT-II); MCF-7; RLE cell lines	*Ex vivo* and *in vitro* Mn assay			TEM, BET surface area analysis, thermal analysis	- Significant dose-dependent increase in Mn in both *in vitro* and *ex vivo* assays after a single administration of MWCNT
Muller et al., 2008 [146]	Heat modified, grinded, or unmodified MWCNT [0.7 μm length]	Wistar rat bronchoalveolar lavage fluid; cultured rat lung epithelial cells)	Mn assay		Histological analysis	BET surface area analysis, SEM, elemental analysis, XPS, Raman spectroscopy, adsorption microcalorimetry	- MWCNT induced acute pulmonary toxicity (granuloma formation) and genotoxicty (Mn): grinded > unmodified > heat modified MWCNT

Pacurari et al., 2008 [149]	Raw SWCNTs [0.8–2.0 nm diameter, 2–5 μm length], Fe 0.07%, Ni 20.6%, Y 6.2%	Normal mesothelial (NM); malignant mesothelial (MM) cells	Comet assay; H2AX phosphorylation	MTT assay; LDH activity; trypan blue staining	Electron spin resonance; protein kinase phosphorylation assay; activation of AP-1 and NF-kB	SEM, TEM, BET surface area analysis, ICP-AES	- ROS-induced: NM > MM. - Dose-dependent decline in cell viability in both cell types. - Significant NF-κB activation, increased DNA damage and H2AX phosphorylation at 25 and 50 μg/cm^2 in both cell lines. - DNA repair was impaired in MM.
Zeni et al., 2008 [131]	SWCNT, >90% purity [1.1 nm diameter, 50 μm length]	PBL	Comet assay	LDH and caspase-3 activity; resazurin assay		None	- Decrease in metabolic activity and cell growth at highest concentrations (25 and 50 μg/mL). - No cell death, apoptosis or DNA damage detected.
Kisin et al., 2007 [148]	SWCNT, 99.7% purity [0.4–1.2 nm diameter, 1.3 μm length]	Chinese hamster lung fibroblasts (V79); *Salmonella typhimurium* (YG1024, YG1029)	Comet assay; Mn assay; Ames test	Trypan blue staining		ICP-AES, Raman spectroscopy, NIR spectroscopy, BET surface area analysis, SEM, TEM	- Comet assay: DNA damage after 3 h, 96 μg/cm^2. - Mn assay and Ames test: no significant increase in DNA damage (up to 96 μg/cm^2, 24 h)
Zhu et al., 2007 [147]	MWCNT	MES	Double strand break repair protein (Rad51 and XRCC4) assay; adenine phosphor-ribosyltransferase assay		Alkaline-phosphatase (AP) detection	None	- Cellular apoptosis and activation of p53 within 2 h of exposure. - Increased expression of 2 isoforms of the base excision repair protein OGG1. - Increased mutation frequency.
Ecogenotoxicity							
Vevers and Jha, 2008 [152]	TiO_2	*In vitro*: RTG-2 fish cells	Comet assay (+/− UV-A light); Mn assay	Neutral red assay	+/− fpg for ROS damage	TEM, SEM, OD reading for aggregation	- No chromosomal damage (Mn). - Comet assay: fpg treatment and UV-A co-treatment showed more DNA damage in NP exposures than controls.
Reeves et al., 2008 [151]	TiO_2	*In vitro*: Gfsk-S1 fish cells	Comet assay (+/− fpg enzyme; +/− UV-A)	Neutral red assay	Electron spin resonance	None	- No toxicity up to 1000 μg/mL (only evident in conjunction with UV-A). - Fpg sensitive sites at 1, 10 and 100 μg/mL. Without fpg, 100 μg/mL showed DNA damage. - UV-A co-treatment increased DNA damage further.
Gagne et al., 2008 [211]	Quantum dots (CdTe)	*In vivo*: mussel gills, digestive glands and gonads	Alkaline DNA precipitation assay			ICP-AES, filtration for size	- Oxidative stress induced at 5.6 μg/mL. - Reduced DNA damage in treated mussels compared to controls at 4 and 8 μg/mL.

DLS, dynamic light scattering particle sizing; ICP-AES, inductively coupled plasma atomic emission spectrometry; NIR, near infra red spectroscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy; and XPS, X-ray photoelectron spectroscopy.

Box 1. Glossary.

Abasic sites – site within DNA that is lacking a base
Aneugen – an agent that causes alterations in chromosome copy number within a cell (individual chromosomes may either be lost or multiplied)
Aneuploidy – a state where a cell has gained or lost individual or multiple chromosomes
Apoptosis – programmed cell death, where the cell uses a specialised biochemical mechanism to promote self-destruction
Carcinogen – a substance that is able to promote or initiate cancer
Clastogen – an agent that causes fragmentation or breakage of chromosomes
Cytotoxicity – refers to the ability of a test agent to kill cells
DNA adduct – formation of a complex between a DNA base and a chemical group through covalent bonding
DNA lesion – altered form of DNA or a base that forms following exposure to a genotoxin
DNA repair – the process involving the identification and correction of damaged DNA. Several enzymatic mechanisms are responsible, each of which recognises and repairs specific types of damage
DNA replication – the process of duplicating the DNA within the cells' nucleus, so that when the cell divides, an identical copy of the genome is passed onto each daughter cell
Genotoxicity – refers to the ability of a test agent to induce DNA damage
Genotoxicity assay – a testing method to detect, quantify and characterise the DNA damage induced by a substance under investigation. These methods are indicators for likely carcinogenic agents
Genotoxin – a substance that damages DNA
Mutagenicity – refers to the ability of a test agent to cause point mutations in DNA
Oxidative stress – an imbalance between the generation of harmful reactive oxygen species and the cells' ability to neutralise these oxidants, resulting in damage to cellular components including proteins, lipids and DNA
Point mutation – localised DNA alterations involving only one or a few bases e.g. deletion of base(s) from a sequence, additional bases may be included in the given sequence, or replacement with a different base (known as a base substitution) may occur
Reactive oxygen species (ROS) – oxygen ions and molecules that are highly reactive due to the presence of unpaired electrons. They are formed during normal aerobic cellular metabolism and play an important role in redox signalling and in immune defences, but to prevent excessive cellular damage a balance is maintained by antioxidants that neutralise ROS

Box 2. Commonly used genotoxicity tests.

Ames test

The Ames test, first described in 1972 [194] is used to assess the mutagenic potential of test substances. It uses several strains of the bacteria *Salmonella typhimurium* each of which carries different mutations in various genes, rendering them unable to synthesise the amino acid histidine, thus they require supplemented histidine as a growth supplement. The bacteria are therefore cultured in the presence of the test compound on agar plates lacking histidine and only bacteria that have undergone reverse mutations resulting in the histidine synthesis genes regaining their function (his^+) will survive to grow into colonies. The frequency of colonies formed is proportional to the mutation frequency induced by the test agent at a given dose.

Chromosome aberration test

This assay characterises gross structural and numerical chromosomal alterations induced by the test agent and can be performed on both an *in vitro* and an *in vivo* basis. In the *in vitro* chromosome aberration test, cultured mammalian cells are treated with the test material and then exposed to a chemical that arrests the cell cycle at metaphase, the stage immediately before the replicated chromosomes are separated into two daughter nuclei. The metaphase chromosome preparations are then harvested onto slides, stained with Giemsa (to display the banding patterns on the chromosomes) and the cells are subsequently analysed microscopically for the presence of chromosomal abnormalities based upon their individual structures and number. The *in vivo* version of the assay involves the treatment of rodents with the test material followed by the metaphase-arresting chemical. Metaphase chromosome preparations are made from harvested bone marrow cells and then they are stained and scored as described for the *in vitro* assay. Although this assay is very labour intensive and requires a skilled cytogeneticist to analyse the metaphase preparations, the chromosome aberration test is routinely used to screen for potential mammalian genotoxic carcinogens.

Comet assay

The comet assay, also known as the single-cell gel electrophoresis (SCGE) assay is a versatile, sensitive and rapid method for measuring DNA single- and double-strand breaks at the level of individual cells. The technique can also be adapted for the quantification of alkali-labile sites, oxidative base damage, DNA–DNA or DNA–protein cross-linking and abasic sites [195,196]. Individual cells encapsulated in a thin layer of low melting point agarose gel on a microscope slide are lysed and the DNA is electrophoresed. Under the electric charge, intact DNA moves minimally due to its large size, but if present, small DNA fragments are able to migrate much further resulting in a comet shape with an extended tail drawn out towards the anode (containing the damaged DNA). The DNA is detected following staining with ethidium bromide or propidium iodide. Analysis of the length and fluorescence intensity of the comet tail is directly proportional to the amount of DNA damage [196,197].

Cytokinesis-blocked micronucleus assay

This is a rapid and sensitive method for the quantification and classification of chromosomal damage. Cells that have undergone cell division in the presence of a test substance can be easily identified by using cytochalasin B (an actin polymerisation inhibitor), which blocks the cell cycle at cytokinesis, resulting in binucleated cells. If the test chemical causes chromosomal fragmentation or

loss, then the damaged genetic material lags behind during chromosome segregation and is not included in either of the resulting daughter nuclei. Instead, they are enclosed within a micronucleus and their frequency in binucleated cells gives a measure of genotoxicity induced by the test chemical at a given concentration [198].
To determine whether the micronuclei formed are the result of a clastogenic (chromosome fragmentation) or aneugenic (whole chromosome loss) mode of action, the micronucleus assay is coupled to kinetochore staining. The kinetochores are protein structures that form at the centromere of all chromosomes during nuclear division, thus micronuclei that stain positive for kinetochores contain a whole chromosome and represent an aneugenic event. Whereas those micronuclei that are kinetochore negative are a consequence of a clastogenicity as they contain chromosome fragments. Additionally, nucleoplasmic bridges (a biomarker of chromosome rearrangement) and nuclear buds (a biomarker of amplified DNA) can be scored in the binucleated cells, as can assessment of cytostatic effects, apoptosis and cytotoxicity [199–201].

HPRT forward mutation assay
The *HPRT* (hypoxanthine–guanine phosphoribosyltransferase) forward mutation assay is commonly used as an *in vitro* and *in vivo* somatic mutation test [202]. The *HPRT* gene is located on the X-chromosome, thus loss of function mutants in mammalian male cells (XY) is not masked by the presence of a competent duplicate copy (i.e. are homozygous) and can be easily identified as they confer resistance to the lethal guanine analogue 6-thioguanine [203]. The enzyme encoded by the *HPRT* gene is involved in the salvage pathway for the generation of nucleotides where it is required for the phosphoribosylation of hypoxanthine and guanine, resulting in their salvage for nucleic acid (DNA) biosynthesis. When cells are grown in the presence of the poison 6-thioguanine, the HPRT enzyme will also act on this analogue, enabling the incorporation of it into DNA during replication, leading to the death of normal cells. However, if a mutation arises in this gene following exposure to the agent under investigation, the salvage pathway for nucleotide generation will no-longer function, hence the toxic analogue will not be incorporated into the DNA and viable cell colonies will form. The colony survival frequency therefore indicates the frequency of deleterious point mutations at a given dose.

γ-H2AX staining
In higher eukaryotic cells, the histone H2A variant is phosphorylated on serine 139 in response to DNA double strand breaks (DSB) to form γ-H2AX [204,205]. This phosphorylation is required for DSB signalling and is thought to act as a beacon to recruit and retain DNA repair proteins to the DSB site [206,207]. The presence of γ-H2AX is therefore a sensitive reporter of DNA damage and these sites can be detected by immunofluorescence microscopy utilising fluorescently labelled antibodies specific to γ-H2AX [208].

8-hydroxydeoxyguanosine DNA adducts
Reactive oxygen species generated during oxidative stress can attack DNA and modify the guanine base resulting in the formation of an 8-hydroxydeoxyguanosine (8-OHdG) modification (known as a DNA adduct). This DNA adduct can lead to mutations in DNA when replication occurs as DNA polymerases (which copy DNA) do not recognise it as Guanine. Detection of this DNA adduct can therefore be used as a marker for oxidative DNA damage [209]. Various methods are available for the detection of the 8-OHdG DNA adduct both *in vivo* and *in vitro*. The most sensitive methods used for the detection of this DNA adduct are HPLC and mass spectrometry (GC-MS) based techniques, which are frequently utilised to screen body fluids for oxidative DNA damage. Alternative methods are antibody based techniques such as immunofluorescence, immunohistochemistry or DNA dot blots which involve the detection of an antibody attached to the 8-OHdG adduct.

and the DNA molecule or DNA-related proteins may lead to physical damage to the genetic material. Indeed, it has been shown that nanoparticles of titanium dioxide and silica can enter the nucleus [17,18] where they cause intranuclear protein aggregates that can lead to inhibition of replication, transcription, and cell proliferation [19]. Quantum dots have also been shown to penetrate the nucleus via the nuclear pore complexes [20]. Moreover they subsequently targeted and interacted with histone proteins, but unfortunately in this study the genetic consequence of this intrusion was not investigated.

Alternatively, DNA damage may arise through indirect mechanisms where the nanomaterial does not physically interact with the DNA molecule, but with other cellular proteins such as those involved in the cell division process. Additionally, they may induce other cellular responses that in turn lead to genotoxicity, such as causing oxidative stress, inflammation and aberrant signalling responses (Fig. 3), all of which have been the primary focus of most studies to date and so are discussed in detail in the following section.

2.1. Oxidative stress

A key mechanism thought to be responsible for the genotoxic effects exerted by nanomaterials involves oxidative stress, which refers to a redox imbalance within cells usually as a result of increased intracellular reactive oxygen species (ROS) and decreased antioxidants. ROS are highly reactive molecules that can disturb the homeostasis of the intracellular milieu by reacting unfavourably with cellular macromolecules including DNA, proteins and lipids. ROS-induced DNA damage is typified by single- and double-stranded DNA breaks, base modifications (e.g. formation of 8-hydroxydeoxyguanosine adducts) and DNA cross-links, all of which if un-repaired have the potential to initiate and promote carcinogenesis [21].

Reactive oxygen species are defined as either "primary" or "secondary". "Primary" ROS (e.g. superoxide, $O_2^{\bullet-}$) can be generated through metabolic processes or through the activation of oxygen, which results in the formation of a reactive nucleophilic molecule of oxygen i.e. superoxide anion; this radical does not react directly with DNA or polypeptides. However, they may interact with other molecules such as redox active transition metals (e.g. iron) or enzymes resulting in the production of "secondary" ROS (e.g. $^{\bullet}OH$ radical), which are the primary mediators of DNA damage. In fact, the majority of $^{\bullet}OH$ radicals generated *in vivo* come from the metal-catalysed breakdown of hydrogen peroxide according to the Fenton reaction, whereby $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$ (dismutase reaction), followed by $M^{n+} + H_2O_2 \rightarrow M^{(n+1)+} + {}^{\bullet}OH + OH^-$ (Fenton reaction; with M representing a transition metal) [22].

The transition metals ions (such as cadmium, chromium, cobalt, copper, iron, nickel, titanium and zinc) released from certain nanoparticles have the potential to cause the conversion of cellular oxygen metabolic products such as H_2O_2 and superoxide anions to hydroxyl radicals ($^{\bullet}OH$), which is one of the primary DNA damaging species. Fe(II) can also cause the production of H_2O_2 from molecular O_2, which can diffuse through the cellular and nuclear membrane to react with Fe bound to DNA also resulting in the generation of $^{\bullet}OH$. This in turn

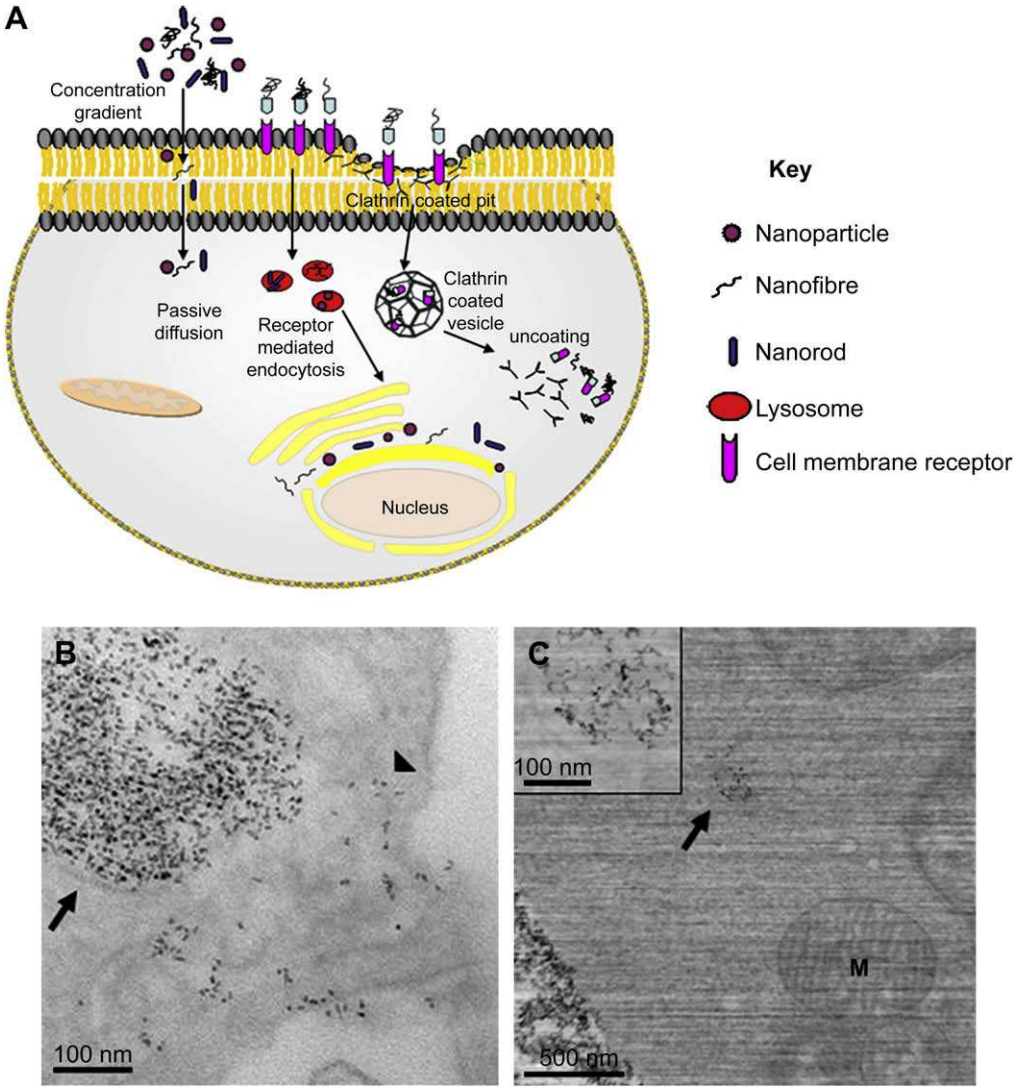

Fig. 2. Cellular uptake of nanomaterials. A. Schematic to illustrate the possible mechanisms of uptake including passive diffusion, receptor-mediated endocytosis and clarthrin- or caveolae-mediated endocytosis; B. TEM image of intracellular CdSe quantum dots with a ZnS shell enclosed within a membrane bound vesicle (black arrow) and free within the cytoplasm as a result of uptake by passive diffusion (black arrow head); C. TEM image of dextran-coated iron oxide nanoparticles within a membrane bound vesicle (black arrow), and at higher resolution in inset (M = mitochondrion).

can cause Thymine–Tyrosine (DNA–histone protein) cross-links in chromatin [22]. Furthermore, free iron ions can result in $^{\bullet}$OH-induced purine and pyrimidine modifications [23]. Hence, potentially, nanoparticles with an iron component could result in a surplus source of iron within the cells fuelling the generation of highly reactive hydroxyl radicals via the Fenton reaction.

In addition to composition (and thus the presence of transition metal catalysts), the high surface area associated with nanomaterials can promote the generation of ROS. Consequently, the smaller the nanoparticle, the higher the oxidative stress they induce [24,25]. There have been numerous studies demonstrating the induction of ROS following exposure to nanoparticles [26–28]. In the case of titanium dioxide nanoparticles (TiO_2 NPs), Park and colleagues [29] demonstrated that their perinuclear distribution correlated with the induction of ROS in the same region (as visualised by a fluorescence reporter).

Oxidative DNA attack has been shown by many studies to govern the genotoxic effects of the nanoparticles as determined by the comet assay and micronucleus assay [26–28]. Furthermore, oxidative stress activates specific signalling pathways including mitogen activated protein kinase (MAPK) and NF-kB [30], which together with the depletion of antioxidant defences, lead to the release of pro-inflammatory cytokines [31]. The overall result of this signalling cascade is the trigger of inflammation, a defensive reaction that leads to further ROS release from inflammatory cells (e.g. neutrophils), resulting in a vicious circle of events that is also central to the pathogenic consequences of particle exposure [32,33].

2.2. Inflammation

Inflammation is an important physiological process in response to tissue injury and is mediated by inflammatory cells that secrete a large variety of soluble factors, including cytokines (e.g. interleukins (IL) and tumour necrosis factor (TNF) protein families), migration inhibition factors, reactive nitrogen species and ROS. Though these factors are important protective defences against infection and/or tissue injury, they can also promote DNA damage in the form of chromosomal fragmentation, point mutations and the formation of DNA adducts; additionally, they inhibit DNA repair and induce aberrant methylation patterns leading to altered gene

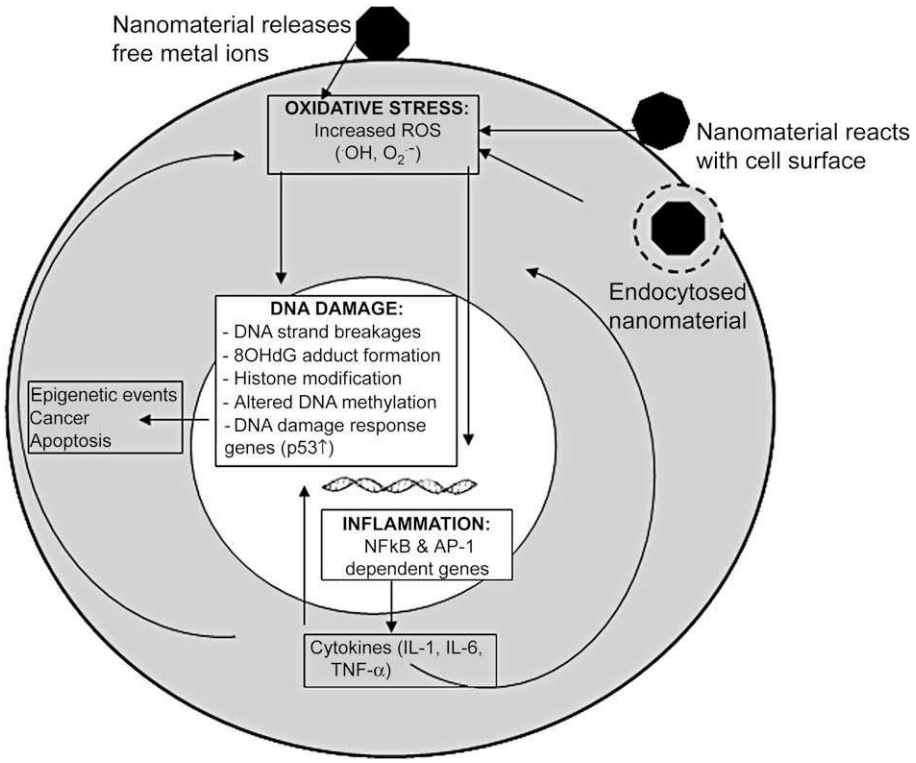

Fig. 3. Indirect mechanisms that can lead to genotoxicity. Nanomaterials may result in oxidative stress or inflammatory responses that in turn have the potential to damage DNA and alter transcriptional patterns.

expression profiles [34,35]. Consequently, chronic inflammation has been strongly associated with carcinogenesis [36–39].

To date, there are many studies demonstrating that nanomaterials can exhibit inflammatory responses. Their small size and thus large surface area appear to be centrally involved in promoting inflammation; indeed studies have shown that ultrafine carbon black and TiO_2 NPs are also associated with greater inflammatory potency in the lungs of rats following intratracheal instillation, as compared to their fine counterparts [40,41]. However, the role that particle composition has to play might be an important consideration as it influences the degree of the inflammatory response induced. For example, *in vitro*, in inducing inflammation, cobalt > silica > TiO_2 > zinc oxide nanoparticles, whilst induction of inflammatory markers was absent following nickel and iron oxide nanoparticle exposures [29,42,43]. Thus, some nanomaterials clearly have the potential to induce oxidative DNA damage through the excessive formation of ROS, not only through corrosion leading to the release of metal ions, but also as a result of chronic inflammatory responses.

2.3. DNA damage responsive signalling

Nanomaterials have been shown to cause oxidative stress, an internal metabolic event that can induce DNA damage. This damage can invoke various cellular responses such as cell cycle arrest, apoptosis and importantly, DNA repair. Since DNA damage has been implicated in carcinogenesis, DNA repair is therefore essential in order to maintain genetic integrity and cell survival. But if repair fails to occur during or before replication of the damaged DNA it could potentially lead to mutagenic and therefore carcinogenic consequences.

When DNA is damaged a key effector molecule that is activated is p53. This tumour suppressor gene has been described as 'the guardian of the genome' as it is responsible for arresting the cell cycle and activating transcription of genes that mediate DNA repair, thus preventing the conversion of damage to mutation [44]. However, if the damage present is extensive, then p53 triggers apoptosis in order to eliminate the individual cell for the benefit of the organism. In a recent study, cadmium–telerium quantum dots were found to dramatically increase the activity of p53 (phospho-p53) and consequently, the pro-apoptotic p53-downstream effectors Bax, Puma and Noxa were all up-regulated [45]. Furthermore, Kang and colleagues [46] have shown that nano-TiO_2 can also induce the accumulation of p53 in lymphocytes, in response to DNA damage. Additionally, increased phosphorylation of the DNA damage checkpoint kinases CHK1 and CHK2 at serine 345 and threonine 68 respectively, were also observed in this study.

Altered expression of other DNA damage response genes required to maintain genome integrity has also been observed following nanoparticle exposure. Gold nanoparticles have been shown to down-regulate a number of DNA repair genes including *BRCA1*, *Hus1*, *ATLD*|*HNGS1* and *AT-V1*|*AT-V2* indicating that nanoparticles may interact directly or indirectly with regulators of genome integrity, potentially leading to further genetic destabilisation [47]. Another issue that can contribute to DNA damage-associated repair is the release of metal ions from nanoparticles containing transition metals. For example, Co^{2+} can compete with Mg^{2+} which can impair the ability of repair enzymes to interact with damaged DNA sites, thereby resulting in inefficient correction of the genetic aberrations [48].

DNA repair mechanisms are central elements in preventing genetic damage from being fixed as permanent mutations. When these protective factors are compromised, stable heritable changes may result, thereby increasing the likelihood of cellular transformation and ultimately carcinogenesis.

3. Genotoxicity of nanomaterials

In this section, we review the literature to date that has alluded to the ability of nanomaterials to induce DNA damage. All appropriate studies identified are summarised in Table 1.

3.1. Metal nanoparticles

3.1.1. Gold nanoparticles (Au NPs)

As compared to their bulk counterpart, Au NPs display very different characteristics, for example when smaller than 20 nm, Au NPs are red in colour and they also exhibit catalytic properties [49]. These NPs often become internalised by endocytosis and by 24 h are enclosed in lysosomal bodies arranged around the perinuclear region [50]. Au NPs, 3–8 nm in size have been shown to be non-cytotoxic, nonimmunogenic and reduce ROS and reactive nitrogen species (RNS) in a time-dependent (4 h) and dose-dependent (100 μM) manner in macrophage cells [50]. There is evidence to suggest this antioxidant activity may be due to the ability of Au(1) compounds in inhibiting the DNA-binding activity of AP-1 and NF-κB transcription factors thus in turn down-regulating the expression of pro-inflammatory cytokines, which are involved in the generation of ROS and RNS [51]. However, in contrast, a recent study using 20 nm Au NP on embryonic lung fibroblasts demonstrated significant oxidative DNA damage in the form of 8-hydroxydoxyguanosine (8OHdG) adducts, at concentrations as low as 25 μg/mL Au NP [47]. This was accompanied by decreased expression of DNA repair genes and the cell cycle checkpoint genes MAD2, cyclin B1 and cyclin B2, which is of concern as lowering the cellular DNA damage response pathways, could promote genetic instability, particularly if the cells are subject to further insults.

It therefore appears that despite the inert nature of gold, Au NPs are capable of inducing DNA damage indirectly through an oxidative stress response, albeit in a cell type or size dependent manner. Of importance, was the fact that regardless of the underlying genetic damage and transcription alterations observed by Li et al. [47] in the Au NP treated cells, no cytotoxicity was observed in this study.

3.1.2. Silver nanoparticles (Ag NPs)

Like gold, Ag NPs show different physical characteristics as compared to their larger counterparts; when on the nano-scale they are yellow coloured, demonstrate antibacterial properties and are more potent inducers of apoptosis and inflammation as reflected by phenotypical changes in the liver [49].

Several studies have demonstrated that silver NPs are cytotoxic [52–54], but the reports on their ability to promote oxidative stress are conflicting [49,55] and there is a distinct lack of information on their genotoxic potential. A study comparing the effect of surface coated Ag NPs and uncoated Ag NPs in mouse embryonic stem cells and embryonic fibroblasts has revealed that coated Ag NPs exhibited a more severe DNA damage response indicated by increased expression of repair proteins and H2AX phosphorylation, than uncoated Ag NPs [52]. The authors of this investigation reasoned that uniform and better distribution of the coated particles as compared to the agglomeration of the uncoated particles increased the surface area and provided greater access to the cellular components. However, both types of Ag NPs increased p53 expression and p53 phosphorylation, up-regulated the DNA damage repair protein Rad51 and also elevated the phosphorylation of H2AX at 50 μg/mL, thus indicating that exposure to Ag NP may result in genetic aberrations.

3.1.3. Cobalt nanoparticles (Co NP)

Colognato and colleagues [56] have recently demonstrated that Co NP (100–500 nm) were capable of inducing genotoxicity in human peripheral blood leukocytes. They showed a dose-dependent increase in the frequency of micronucleated lymphocytes, with the first statistically significant increase arising at 40 μM cobalt nanoparticles (48 h incubation) and this was accompanied by reduced cell viability.

Given that the fate of Co NP once internalised in cells is unknown, there is the possibility that they may be corroded over time, releasing Co^{2+} ions and it is therefore important to consider the biological effect of these ions as well as the NP themselves, which warrants the need for additional long-term experiments. Indeed, it has been demonstrated that cobalt ions can produce genotoxic responses typified by single strand breaks, chromosomal aberrations, sister-chromatid exchanges and covalent DNA–protein cross-links through mechanisms involving oxidative stress and the inhibition of DNA repair [57,58]. These studies are vital, as cobalt exposure alone or in combination with other metals particles has been shown to be associated with various lung diseases (interstitial pneumonitis, fibrosis, asthma and lung cancer), that are slow progressing and take time to manifest [59,60]. Furthermore, in a study evaluating the pro-inflammatory effect of different nanoparticles on endothelial cells, higher IL-8 expression was induced by Co NP as compared to silicon dioxide and titanium dioxide, and was thought to be due to the release of Co^{2+} ions from the Co NP, which are known to be potent inducers of IL-8 [43]. As IL-8 is a neutrophil chemo-attractant, this will lead to increased ROS release from the neutrophils and the potential for ROS-induced DNA damage. Hence, the long-term consequence of metallic nanoparticle uptake is certainly an issue that needs consideration.

3.1.4. Cobalt–chromium nanoparticles (Co–Cr NPs)

Most emphasis to date in the field of nanotoxicology has been placed on ambient exposure to nanomaterials, but the possibility of internal exposure from surgical implants also exists and is exemplified by the mechanical wear of orthopaedic joint replacements. Metal-on–metal (MOM) hip implants made from cobalt–chromium (Co–Cr) alloy that are currently in use, have been shown to result in the generation of Co–Cr NPs with an average diameter of 40 nm following erosive wear, and consequently increased levels of metal ions have been reported in patient blood, urine, hair, lymph nodes, bone marrow, liver and spleen demonstrating their dissemination throughout the body [12,14,61]. The International Agency for Research on Cancer (IARC) has classified both cobalt and chromium as human carcinogens separately, but there is little information on the biological consequences of nanoparticulate Co–Cr when internalised.

Co–Cr NPs are not inert; consequently recent studies have emerged indicating that patients with Co–Cr hip replacements demonstrate increased levels of structural and numerical chromosomal aberrations in peripheral blood lymphocytes [62,63]. Furthermore *in vitro* studies have demonstrated that synovial fluid and wear debris obtained from patients undergoing revised hip replacements induced double strand breaks in fibroblast cells [64] and chromosomal damage in primary amnion cells [62]. The literature to date has therefore raised concerns over the carcinogenic risk following mechanical wear of these orthopaedic implants (particularly in young patients receiving such hip replacements) and hence has been addressed by the Department of Health's Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM). However, there is currently insufficient epidemiological data available to determine if there is an association between Co–Cr implants and an increased incidence of malignant disease. Additionally, COM came to the conclusion that the studies to date had not provided convincing evidence for metal specific mutagenic effects and that it was not possible to draw conclusions as to whether the metal ions, interactions

between metal ions, or particulate metals were responsible for the genotoxicity observed (COM/06/S1).

Consequently, *in vitro* studies have been utilised to try and determine the potential mechanism of action and the metallic component responsible for the damage. Co–Cr microparticles (5 μm) have been found to induce cytotoxic and inflammatory responses *in vitro* [12], but it has recently been demonstrated that Co–Cr NPs caused significantly greater levels of DNA damage than their counterpart micron-sized particles in the alkaline comet assay [27]. However, in this study when genotoxicity was assessed using the micronucleus assay, no difference in micronuclei induction was observed between the micro- and nanoparticles. They both induced a dose-dependent increase in gross chromosomal damage that was largely aneugenic, as the resultant micronuclei were centromere-positive. The observed difference between the micronucleus assay and comet assay could be due to differences in incubation time – in the comet assay the authors treated cells for 1 or 3 days, whilst the treatment time was only 12 h with the micronucleus assay. Given that particles may require lengthier treatment times due to their size and mechanisms of cellular uptake, this difference in exposure time may have caused the discrepancy in results between the two assays. Furthermore, assessment of the Co–Cr NPs degree of agglomeration under experimental conditions was not reported, so the true size of the materials exposed to the cells is unknown.

Papargeorgiou and colleagues [27] also reported that the Co–Cr NPs were quick to dissolve once within the cell cytoplasm suggesting that corrosion product(s) may also be involved in the negative cellular responses. Thus it appears that once taken up by cells, the resultant biological consequences may be due to a combination of nanoparticulate interactions together with breakdown products.

3.2. Metal-oxide nanoparticles

3.2.1. Titanium dioxide nanoparticles (TiO_2 NPs)

Nano-sized or ultrafine TiO_2 is used widely in industrial products, pharmaceuticals and cosmetics e.g. in sunscreens to effectively attenuate UV-B radiation [65]. It also has photocatalytic properties that have resulted in the use of TiO_2 NPs as an environment and wastewater disinfectant [66]. Furthermore, TiO_2 NPs have been used as a photosensitizer for the photodynamic therapy of human colon carcinoma cells [67]. Hence, there are currently ever increasing opportunities for both human and environmental exposure to this particular nanoparticle.

Nanoparticulate TiO_2 is considered safe for use in sunscreens by the US Food and Drug Administration (FDA), but there is considerable concern with this ruling as sunlight-illuminated TiO_2 catalyses DNA damage both *in vitro* and *in vivo* [68]. When exposed to UV light, TiO_2 catalyses the generation of reactive oxygen species, such as superoxide anions, hydrogen peroxide, free hydroxyl radicals, and singlet oxygen in aqueous media [69–71]. Once internalised, these nanoparticles have been shown to concentrate in the perinuclear region and interestingly, this distribution correlated with the induction of ROS in the same area [29]. Cai and colleagues [72] found that cytotoxicity associated with TiO_2 NPs to HeLa cells was enhanced in the presence of UV light and suggested this was due to the photo-excited TiO_2 promoting oxidative stress. *In vivo* studies have provided evidence that TiO_2 NPs can cause inflammation, fibrosis, pulmonary damage and even DNA damage [43,73–75], but these studies are limited and *in vitro* studies are required for more mechanistic insight.

Given that oxidative stress and inflammation are associated with inducing genotoxicity via the damaging activity of ROS, it seems likely that exposure to TiO_2 NPs may indirectly result in DNA aberrations and several studies have demonstrated this response. TiO_2 NPs have been shown to result in chromosomal damage in peripheral blood lymphocytes, Syrian hamster embryo (SHE) fibroblasts and in WIL2-NS (a human B-cell lymphoblastoid cell line) using the micronucleus assay [46,73,75]. Rahman and colleagues [75] demonstrated an increase in micronucleus frequency at all concentrations tested (0.5–5.0 μg/cm^2) in SHE fibroblasts. A time-dependent increase in micronuclei was observed from 12 to 24 h with no further increase observed at 48 h and 72 h suggesting saturation. To determine whether the micronuclei were the result of a clastogenic (chromosomal fragmentation) or aneugenic (whole chromosome loss) mode of action, kinetochore staining was used and the results demonstrated that TiO_2 NPs caused clastogenicity, consistent with ROS-related damage. However, physico-chemical characterisation of the TiO_2 material used in this study was lacking which limits the assessment of the results. The study by Wang and colleagues [73] also found an increased micronculeus frequency, at 65 μg/mL, using the *hprt* forward mutation assay and comet assay to demonstrate that TiO_2 NPs are also capable of inducing point mutations and DNA strand breakages. This observation was later reinforced by Kang et al. [46] and Karlsson et al. [26], who both observed increased strand breakages by the comet assay following the exposure of TiO_2 NPs to lung epithelial cells. While a recent study demonstrated that TiO_2 NPs were clearly mutagenic, and that this genotoxic response was suppressed by nitric oxide synthase and COX-2 inhibitors, suggesting that RNS (particularly peroxynitrite anions) were linked to TiO_2 mutagenicity [76].

The damage observed in all these studies is consistent with that caused indirectly by oxidative stress. However, there are conflicting reports such as that by Theogaraj and colleagues [77] who did not find that TiO_2 NPs were genotoxic; using the chromosomal aberration test in CHO cells, no increases in DNA damage frequency were observed in either the absence or presence of UV light. Nonetheless, it is worth noting that the cellular responses elicited by TiO_2 NPs are highly dependent upon size and form. For example, TiO_2 anatase NPs up to 20 nm in size have been shown to induce chromosomal damage (10 μg/mL) through increased micronculeus frequency, whilst in the same study such genotoxicity was not induced by 200 nm anatase or 200 nm rutile TiO_2 NPs [28]. Unfortunately, in many studies, this degree of detail on the material under investigation is lacking, thus making it difficult to compare the results and gain a definitive understanding of the TiO_2 NPs and the conditions of exposure that lead to DNA damage.

3.2.2. Zinc oxide nanoparticles (ZnO NPs)

ZnO NPs are used in a variety of different applications including cosmetics, paints, as drug carriers and fillings in medical materials [78]. They are also used as UV blocking materials, especially for light in the UV-A region, but their high catalytic activity in oxidation and photochemical reactions restrict their use as UV blockers [79].

Though ZnO NPs are believed to be nontoxic and biocompatible [80] there are currently a small number of reports in the literature demonstrating that they could exert negative cellular responses. Exposure to ZnO NPs has been associated with inflammatory responses [9] and cytotoxicity [81], but there is only one study that has considered the DNA damaging potential of ZnO NPs. Dufour and colleagues [78] used the chromosome aberration test on CHO cells to examine genotoxicity in the dark, and under pre-irradiation and simultaneous UV irradiation conditions. In this investigation, chromosomal aberrations induced by ZnO NPs with a mean diameter of 100 nm, were enhanced by UV light, with increased clastogenicity under pre-irradiation (PI) and simultaneous irradiation (SI) conditions than in the dark. The lowest dose tested that resulted in a statistically significant increase in DNA damage was 54 μg/mL under both PI and SI conditions, but was higher at

105 μg/mL when exposure was performed in the dark. Hence, this study indicates that ZnO NPs may cause photo-genotoxicity, but again there was a lack of information on the physico-chemical features of the sample under investigation. Thus, there is clearly a need for further assessment to fully classify the genotoxicity of these particles with more focus on the dependence of size and physico-chemical characteristics.

3.2.3. Silica nanoparticles

Silica induces inflammatory (NF-κB activation) and oxidative stress responses both *in vivo* and *in vitro* [9,82,83], but cytotoxicity is largely only observed at high concentrations [84,85]. Additionally, silica nanoparticles have been shown to enter the cell nucleus where they could potentially bind to the DNA phosphate backbone [19]. Since the silica nanoparticles can result in increased ROS levels [83] and given that the hydroxyl radical is a highly reactive molecule the generation of $^{\bullet}OH$ close to the DNA could readily lead to the induction of DNA strand breaks and oxidised bases [22] which could have important implications in the development of cancer.

Silica nanoparticles also have an impact on nuclear integrity by forming intranuclear protein aggregates that can lead to inhibition of replication, transcription, and cell proliferation [19]. Additionally, they have been shown to reduce replication activity down to 67% and 60% after 6 and 24 h respectively and transcriptional activity down to 82% after 4 h, thus resulting in decreased cell proliferation after 24 h (88%) and 48 h (65%). This study underscores the importance of nanoparticles sizing since particles >200 nm fail to penetrate the nucleus, do not alter nuclear structure and function, and also do not interfere with gene expression [19].

Despite these abnormalities, there is limited evidence to suggest silica nanoparticles are genotoxic and some recent studies utilising the comet assay have demonstrated that silica nanoparticles ranging in size from 20 to 400 nm do not exert significant genotoxicity [85,86]. In contrast, one investigation based on the micronucleus assay found that these nanoparticles do indeed induce chromosomal damage [87]. Thus, to get a clear indication of genotoxic potential a battery of standardised tests that quantify different types of genetic aberrations are required to cover all potential forms of DNA damage that may be induced following exposure to nanoparticles.

3.2.4. Iron oxide nanoparticles

Iron has long been associated with cancer and several pathways for iron-induced carcinogenesis have been suggested based upon oxidative stress causing lipid peroxidation and direct damage to DNA and proteins [22,88–90]. Spindle cell sarcoma and pleomorphic sarcoma in rats have been associated with iron-overload following intra-muscular injections of iron–dextran complex [91]. It is possible that these neoplasms could be the result of a phenomenon known as solid-state carcinogenesis, whereby implantation of a solid foreign body leads to tumour formation. However, when in a complex with dextran, iron is dispersed in solution and thus is unlikely to be associated with this affect. However, it is possible that their enhanced solubility results in increased cellular uptake. Consequently, overloading cells with iron oxide nanoparticles may have negative cellular influences by resulting in a surplus source of iron within the cells, fuelling the generation of highly reactive hydroxyl radicals via the Fenton reaction.

Iron oxide nanoparticles have attracted much attention not only because of their superparamagnetic properties but also because they hold great potential in many biomedical applications such as drug delivery, magnetic resonance imaging (MRI) contrast enhancement, and in the targeted destruction of tumour tissue through hyperthermia. Uncoated iron oxide nanoparticles have very low solubility, thus to be used effectively for any clinical application and to improve biocompatibility and biodistribution, iron oxide nanoparticles are coated with amphiphilic coatings such as polyethylene glycol (PEG), dextran or dendrimers [92–94]. They can also be bound to complex biological molecules such as antibodies, peptides, hormones or drugs [95]. This derivitisation of iron oxide plays an important role in internalisation efficiency and cytotoxicity [96–98]. However, the stability of these coatings is largely unknown, as are the consequences of their breakdown in the internal cellular environment. Reports are now starting to emerge suggesting that this may be a significant problem associated with these nanoparticles.

Dextran-magnetite (Fe_3O_4) nanoparticles have been shown to result in cell death and reduced proliferation similar to that caused by uncoated iron oxide particles [93]. The reason behind the observed cytotoxicty with dextran-magnetite was attributed to breakdown of the dextran shell exposing the cellular components to chains or aggregates of iron oxide nanoparticles. However the cell behaviour and morphology of cells treated with dextran-magnetite was different from the uncoated nanoparticles, with the former showing more prominent membrane disruptions [99].

Stroh and colleagues [100] noted that citrate-coated very small superparamagnetic iron oxide nanoparticles (VSOP) can lead to cellular oxidative stress in rat macrophages as shown by a significant increase in the levels of malonyldialdehyde and protein carbonyls. Interestingly this increase was only transient, as 24 h post-incubation resulted in a decrease to control levels. This increased oxidative stress was however, eliminated by the iron chelator desferal and the intracellular spin trap PBN suggesting iron may have been released from VSOP and was responsible for the effects observed.

Auffman et al. [101] have suggested that a coating such as meso-2,3-dimercaptosuccinic acid (DMSA) that prevents direct contact between maghemite (Fe_2O_3) nanoparticles and human dermal fibroblasts can inhibit a potential genotoxic effect. They showed a significant decrease in cell viability of fibroblasts following 2 h and 24 h exposure to DMSA-coated maghemite (NmDMSA) at concentrations between 10^{-6} and 10^{-3} mg/mL. Interestingly, the higher concentration of 0.1 mg/mL did not show reduced cell viability, but did result in increased metabolic mitochondrial activity. This finding could be attributed to the increase in aggregate size from ~30 nm to ~70 nm at higher concentrations resulting in less effective contact between NmDMSA and the cells. Genotoxicity (using the comet assay) was assessed in this study, but was only observed at the higher concentrations of 10–100 μg/mL. Furthermore, an *in vivo* study on Swiss mice using magnetite NP coated with polyaspartic acid, showed a time- and dose-dependent increase in micronucleus frequency [95]. Thus, it appears that genotoxic responses may be in part dependent upon coating, but the differences observed in the literature may also be due to limitations with regards to the genotoxicity assays used to study the materials.

3.3. Quantum dots (QD)

QD are semiconductor nanocrystals that have highly desirable optical properties, which promise to revolutionise bioimaging. However, the main drawback with these nanoparticles that is likely to hinder their use *in vivo*, is the fact that they consist of a core comprised of highly toxic elements such as cadmium (Cd), tellurium (Te), selenium (Se), and lead (Pb). Given that QD have been found to degrade under photolytic and oxidative conditions, these dangerous materials could be freely released in the oxidative cellular environment [102]. Consequently, QD are now largely designed with a cap/shell (e.g. made of zinc sulphide) surrounding the metallic core and functional groups or coatings such as PEG are

also utilised to render the QD hydrophilic hence improving their biocompatibility and bioactivity. In fact, a wide range of coatings can now be employed to give QD highly specific bioactivities for diagnostic (e.g. imaging) and therapeutic (e.g. drug delivery) purposes. However the stability of these coatings is a very important issue, because if they are degraded or broken down then the composite metal core may be exposed.

There are now several studies in the literature demonstrating the protective effects that zinc sulphide (ZnS) shells confer on QD, significantly reducing toxicity as compared to the uncoated counterparts [103,104]. However, the choice of shell or coating material needs to be carefully selected as some have themselves been found to be (geno)toxic and purification of the material is another important factor. For example, Hoshino and colleagues [105] revealed that crude QD coated with carboxyl groups (QD-COOH) prepared only by membrane filtration showed stronger DNA damage than purified QD-COOH (prepared by ultrafiltration) after 2 h incubation, indicating residual contaminants from the fabrication process were responsible for the genotoxicity. Although almost all the damage induced by purified QD-COOH and not by crude QD-COOH was repaired after 12 h, this does not rule out the presence of some non-repaired DNA lesions, which could lead to gene mutations and/or chromosome aberrations. Further investigation showed that the ingredient mercaptoundecanoic acid, used in the preparation of QD-COOH was highly damaging. Thus, QD fabrication processes and surface coating properties are fundamental to understanding their toxicity.

Although studies on QD with coatings such as ZnS address safety issues regarding their biocompatibility in biomedical applications, it is important to consider that if QD are retained in the body for long periods of time their coating might be degraded releasing the QD-core, which could then induce significant cellular damage. Uncoated CdTe QD are associated with elevated ROS production that damages the plasma membrane, mitochondria and nucleus, and in these cells antioxidant enzymes including superoxide dismutase 1 (SOD-1) are up-regulated in an attempt at cellular protection [45,106]. However, coated QD can also cause the generation of free radicals and thus oxidative damage [107].

With regards to genotoxicity, two studies have demonstrated that CdSe/ZnS quantum dots cause DNA fragmentation and nicking in cell-free systems [107,108]. Additionally, there are a number of investigations that have reported nuclear perturbations and in some cases, interaction with DNA. Negatively charged CdTe and CdSe/ZnS QD enter cells through endocytosis and become enclosed in endosome-like vesicles, which transport them to the perinuclear endoplasmic reticulum membrane. Interestingly, CdTe QD have been shown to enter the nucleus via nuclear pore complexes and to also penetrate the nucleoli of macrophages where they specifically target positively charged histones [20]. Additionally, QD such as CdTe possess unique photophysical properties that induce global hypoacetylation of histone-3 with increasing concentration of QD treatment [45]. CdTe can also induce nuclear shrinkage, chromatin condensation and loss of mitochondrial cristae [45]. Thus, there is evidence for nuclear interaction, but again few studies have specifically focussed on the genotoxic potential of QD using a battery of tests to determine their mode of action according to physico-chemical characteristics.

3.4. Fullerenes

Fullerenes are a set of nanomaterials that have a caged structure, consisting purely of carbon. Their presence is widely reported in the environment as imperfect combustion products from fuels including pine root, tung oil, rapeseed and in soot from pine wood and toluene manufactured in laboratories [109]. They have also been discovered in geological samples billions of years old [110], and currently, they are being used in the cosmetics industries (in anti-aging creams) and are also under development for use in the clinical domain.

Research on these nanomaterials has deemed them to be less toxic than carbon nanotubes, carbon black and diesel exhaust particles [111]. Several studies have demonstrated fullerenes are free radical scavengers with antioxidant properties [112–114]. However, they do appear to have an ability to both quench and conversely, to generate ROS [112], with evidence demonstrating that fullerenes are able to damage DNA possibly as a result of oxidative stress-based mechanisms. Fullerenes can form complexes with DNA (in cell-free systems), induce DNA strand breakages, mutagenicity and chromosomal damage, with chronic 80 day exposures at very low concentrations of 1 ng/mL resulting in an increased micronucleus frequency in CHO, Hela, HEK293 and MEF cells [76,115–118].

Although the above studies have reported DNA damaging responses following exposure to fullerenes, there are conflicting reports in the literature, where no such damage has been observed [119,120]. An example is the recent study by Jacobsen and colleagues [111], where fullerenes did not induce significant increases in DNA strand breakages as detected by the comet assay, whereas Dhawan et al. [115] did report positive genotoxic responses using the same test. The discrepancies in these investigations may be due to a number of factors including exposure time, nanomaterial preparation, cell type, and given that in all these studies no physico-chemical characterisation was detailed (Table 1), it is difficult to directly compare the results.

3.5. Fibrous nanomaterials

Carbon nanotubes, nanofibres, nanowires and nanorods are a promising breakthrough in the field of material science for consumer, industrial and medical purposes. The rise in the current and the expected near-future interest in their use means that the extent of exposure to these materials is likely to increase. However, due to the fibrous structure of these nanomaterials, parallels have been drawn with asbestos and consequently it is possible that nanotubes, nanofibres, nanowires and nanorods might fit the fibre toxicological paradigm whereby toxicity is based on a high-aspect ratio and biopersistance, leading to prolonged inflammatory responses.

To date, there are very few studies in the literature that have focused on the cellular impact of nanofibres, nanorods or nanowires. A number of reports demonstrate that nanorods such as fluorescent lanthanide ortho phosphate and ZnO nanowires demonstrate no significant toxicity based largely on cell proliferation and apoptosis assays [121–123]. However, these studies in some aspects are very limited and the data is often inconclusive due to study design. For example, a recent publication reports that ZnO nanowires were not toxic as they broke down into zinc ions in the presence of horse serum, but no cellular-based toxicity studies were performed [123]. In fact a study on mice demonstrated that lung cell injury and inflammation have been attributed to a soluble component, possibly zinc metal ions in atmospheric dust, through the induction of Type 1 necrosis in alveolar epithelial cells followed by inflammation [124]. Whilst another study by Driessen and colleagues [125] showed that the IL-1β, cytokine was stimulated by zinc ions.

The safety evaluation of nanofibres, nanowires and nanorods of differing compositions requires considerably more attention to fill in the current knowledge gaps. In contrast, carbon nanotubes (CNT)

have attracted considerably more attention, but despite this, the literature is still conflicting with some investigations reporting cytotoxic effects following the exposure of several cell types to both single walled carbon nanotubes (SWCNT) and multi walled carbon nanotubes (MWCNT) [126–131]; whilst others demonstrate very low or insignificant cellular responses [132–135]. Additionally, few have considered the genotoxic potential of CNT, which is of importance given that asbsestos fibres are considered genotoxic as they promote structural and numerical chromosomal damage, together with point mutations [136–140]. Given SWCNT have been found to not only penetrate cells, but can also become localised in the nucleus, assessment of their genotoxic potential is of key concern [141]. Furthermore, recent publications have demonstrated that MWCNT not only induce an asbestos-like inflammatory response and the formation of granulomas *in vivo* following intraperitoneal administration [142], but also promote the development of mesothelioma in more long-term *in vivo* studies following the administration of a single intrascrotal or intraperitoneal dose [143,144]. These investigations therefore demonstrate that MWCNT have carcinogenic potential, but the underlying mechanism has not been studied and remains unknown.

A small number of reports have highlighted the potential genotoxic impact of CNT (Table 1), several of which have found that MWCNT induce dose-dependent increases in chromosomal damage (both aneugenic and clastogenic events) and point mutations [145–147], which might be responsible for their carcinogenicity. In contrast, the literature is currently conflicting with regards to SWCNT. Two studies have reported that high purity SWCNT of very different lengths (1 μm versus 50 μm) did not cause DNA strand breakages or increases over background mutation frequencies at doses up to 100 μg/mL [111,131]. However, there are another two studies that have observed the opposite using the same technique (the comet assay). Kisin and colleagues [148] found that high purity, 1.3 μm length SWCNT induced significant DNA strand breakages at 96 μg/cm^2 following 3 h and 24 h exposure using the Comet assay. Pacurari and colleages [149] also found significant DNA damage but at lower concentrations of 25 μg/cm^2 using SWCNT with lengths of 2–5 μm and approximately 72% carbon purity. Interestingly, in this study ROS scavengers only moderately reduced DNA damage therefore indicating that although oxidative stress may be an indirect genotoxic mechanism of action for SWCNT, there are other (as yet unknown) mechanisms involved too.

It could be argued that the increased DNA damage observed by Pacurari and colleagues [149] could have been due to the high content of metal impurities in the CNT assessed. However, in this study metal chelators only partially reduced the oxidative stress induced following exposure to their SWCNT sample, suggesting that the nanotubes themselves were also a source of ROS generation as opposed to the metal impurities alone. Thus the inconsistencies between studies in the literature may be due to the use of different model cell lines (Table 1), variable means of dispersing the CNT or differences in the physico-chemical characteristics of the nanomaterials examined under the experimental conditions used in each of the studies, which often are not fully described. From the research to date into CNT toxicity, it is becoming increasingly evident that their physico-chemical characteristics are likely to be central in governing the resultant biological response following exposure. Additional factors such as rigidity are an important characteristic that will influence the final form that the CNT will take under experimental conditions and thus their cellular impact [133]. Surface chemistry is also another key parameter that is often overlooked, but has been reported to have a substantial effect upon genotoxic potential [146]. Thus, emphasis on the need for complete physico-chemical characterisation is critical in aiding the interpretation and comparison of results in the literature.

3.6. Eco-genotoxicology

DNA damage induction by nanomaterials is not just relevant to the human species. Whilst human exposure to genotoxic nanomaterials might lead to the induction of cancers or of genetic abnormalities in our offspring, it is also important to consider the effects of nanomaterials to the genetic components of environmental species. It is conceivable that the environment might be exposed to greater levels of nanomaterials than we humans are, due to the contamination of water supplies surrounding manufacturing plants. Hence, monitoring key environmental species for DNA damage is necessary to prevent deleterious effects on our indigenous wildlife leading to species extinction in extreme cases. Very little data is currently available to assess the overall toxicity of nanoparticles in environmental species [150]. In particular, the threat of DNA damage is of concern as this may lead to heritable abnormalities and hence effects on fitness in these ecosystems, but these studies are especially lacking [4].

Another important reason to monitor environmental species for toxicity (including genotoxicity) resulting from the nanotechnology industries, is that this can inform us about the potential threats posed by some of these nanomaterials to human health. Hence, using "sentinel species" for DNA damage induction can identify those exposures that are more worrisome, which may be directly applicable to the human context.

As well as large numbers of studies on toxicity to environmental species, there have been a few studies which have assessed DNA damage induction by nanomaterials in environmental species. In particular, these studies have assessed TiO_2 NPs and QD *in vitro* in fish cells, or in whole mussels respectively. The *in vitro* studies of TiO_2 NPs have used the micronucleus assay and the comet assay and have shown some DNA damage (at doses of 1, 10 and 100 μg/mL) in the comet assay, which were exacerbated by UV-A exposure, suggesting that photo-genotoxicity may be important [151]. The DNA damage shown in these cells was only found when fpg (an enzyme that cleaves oxidatively damaged DNA) was included. Without fpg, only the highest dose gave a positive response. Hence, this supports the concept that the DNA damage induced by TiO_2 NPs was generated indirectly through ROS intermediates. Interestingly, using the micronucleus assay, no DNA damage was seen [151,152]. The *in vivo* study in the mussels exposed to CdTe QD showed some evidence of ROS induction, but actually showed less DNA damage compared to the untreated animals. Hence, it is possible that in this instance CdTe QD protected the cells in the gills, digestive glands and gonads from background levels of DNA damage. Obviously, more studies are needed to confirm these findings.

There are clearly only a few datasets available in environmental species presently and more data is required to determine the impact of nanomaterials on the environment. Given the likely exposure routes through contamination of the waterways by industrial manufacturing sites, it is particularly important to assess the effects of nanomaterials to the DNA of aquatic species (freshwater and seawater). As with the studies in mammalian cells presented elsewhere in this review, the existing studies in the environmental species suffer from a lack of thorough particle characterisation. Furthermore, studies *in vivo* are more complicated due to the need to ensure that true exposures have occurred in the target tissue. There is indeed a great need to be able to monitor the level of nanomaterials reaching the tissues and cells and correlating this with the level of any toxicity and DNA damage. This is particularly true where internal assessments are carried out *in vivo*.

4. Physico-chemical characterisation: factors influencing (geno)toxicity

The toxicokinetics of nanomaterials are still not well understood, but it is becoming increasingly evident that their physicochemical features play a central role in governing their cellular uptake and subsequent physiological consequences. Currently there is some uncertainty about which parameters have the most influence over (geno)toxicological responses, but the evidence accumulating points to a host of different factors that all appear to be involved in modulating biomolecular interactions.

4.1. Size, shape and surface area

The sub-100 nm size of nanomaterials is one of the primary features that provide them with unique properties over larger materials of the same composition, because with decreased size, the number of particles per unit mass increases. However, their size can also represent a health hazard as they may be more likely to cross biological barriers, gaining entry to the body and subsequently size governs their kinetics including absorption, distribution, metabolism and excretion. Once inside the body the nanomaterials may then be small enough to readily enter cells and their size may promote interactions with biomolecules which has the potential to destabilise normal cellular functioning. In one respect this could be advantageous and is the driving force behind the development of new nanomedicines and nanodevices for clinical healthcare [15,16]. On the other hand however, such interactions following exposure to engineered nanomaterials in the workplace or from consumer products may have negative effects upon human health.

With regards to cellular uptake, size can be a critical feature. There are several different mechanisms by which nanomaterials can be internalised (Fig. 2), perhaps the most prominent being diffusion across the plasma membrane (either directly across the membrane or through membrane channels 10–30 nm wide) or by endocytosis, an energy dependent mechanism that can involve a number of different routes. If nanomaterials are of the correct size and shape, they may dock on membrane receptors facilitating receptor-mediated endocytosis. Alternatively, clathrin- or caveolae-mediated endocytosis may occur, which result in the formation of pits in the region of 120 nm or up to 80 nm respectively that regulate the size of the material they are able to enclose [153]. In fact, investigations using a range of nanoparticle sizes have demonstrated that small nanoparticles up to 200 nm are primarily internalised by both clathrin-and caveolae-mediated endocytosis and that 50 nm particles are taken up at the fastest rate and to the greatest extent when compared to both smaller (down to 14 nm) and larger (up to 500 nm) particles [154–157].

There are several studies that clearly demonstrate that size does matter with respect to toxicological outcome, many of which have focused on carbon black, ZnO and TiO_2 NPs versus microparticles. In general, inhalation studies have demonstrated that the nanoparticles penetrate deeper into the lungs and become localised within many cell types, they demonstrate a greater inflammatory response and are often associated with increased toxicity as compared to their fine sized counterparts [17,39,158–161]. Although genotoxicity was not considered in these studies, given the well established relationship between chronic inflammation, DNA damage and carcinogenesis, these reports give rise to substantial cause for concern with regards to the long-term health implications following nanoparticle inhalation.

Another issue related to adverse effects of nanomaterials on cellular systems is their shape or morphology. There are very few studies focusing on the toxicological relationship associated with this parameter alone, but one recent publication by Muller and colleagues [146] demonstrated that removal of structural defects from MWCNT was sufficient to substantially reduce their inflammatory response and overall toxicity. Additionally, a number of studies have found that the shape of a nanomaterial can highly influence their rate of uptake. Spherical nanoparticles show higher uptake than nanorods, while internalisation of these cylindrical shaped materials is strongly influenced by their dimensions such that high-aspect ratio particles are internalised considerably faster than low-aspect ratio particles with more symmetry [155,156]. In addition to governing cellular uptake, size and morphology are key determinants in the surface area associated with a given mass dose.

As particle size decreases, the number of particles per unit of mass and thus the overall surface area will increase. The shape of the nanomaterials will also contribute to the overall surface area, with spheres having slightly smaller surface areas than an octagonal shaped structure of the same size. This larger surface area enhances the catalytic activity of the material and thus has been well reported to increase its reactivity because surface atoms have a tendency to have unsatisfied high energy bonds. In order to gain stabilisation, these surface bonds will readily react with other molecules, hence the larger the surface area, the greater the materials' reactive potential [40]. If these nanomaterials are therefore able to gain access to the cellular environment, there is a much greater chance that the enhanced surface area as compared to counterpart micron-sized particles will result in interaction with biomolecules, causing direct cellular damage and promoting oxidative stress.

4.2. Purity

Nanomaterial purity is also an important consideration as residual contaminating metals may actually be responsible for (geno)toxicological responses rather than the actual nanomaterial itself, the quantity of which are dependant upon the synthesis procedure employed. Although post-production processing removes most of these metal catalysts, even purified nanomaterials may still contain up to 15% residual metal by mass. While researchers have made encouraging attempts to purify the nanomaterials under investigation in order to rule out the effects of impurities on the observed toxicity, often the damaging effects of the purification process have been overlooked.

The best examples of the consequences of impurities in relation to nanomaterial toxicity have been described for carbon nanotubes, where the purification methodologies utilised involve washing or ultrasonication with dilute acid. However, these methods have been shown to destroy the nanomaterials, even introducing defects and modifications to their surfaces through the addition of functional groups that may affect their overall biological impact [162–164]. Furthermore, the purification process is thought to oxidise the nanomaterials and might also break carbon nanotubes into shorter lengths [165].

The most commonly observed carbon nanotube impurities can be divided into three categories: metals, organic materials, and support material which are most commonly; Co, Fe, Ni, and Mo as single metals or mixtures [166]. These metals are considered contaminants as they do not play an active role in the nanomaterials' function, but this is not always the case. For example, there is current research underway into the synthesis of CNT rich in lead metals specifically for X-ray protection shields. However, where metals are present as impurities, iron is one of the primary sources of damage as it induces oxidative stress through Fenton or Haber–Weiss reactions [149,167–170]. Indeed, iron contaminants in CNT have been shown to result in a substantial loss of glutathione and increased lipid peroxidation in alveolar macrophages,

indicators of oxidative stress [171]. Yet there is still conflict in the literature as some studies, such as that by Lam and colleagues [172] demonstrate that SWCNT induce lung lesions (granulomas) in a dose-dependent manner in mice, regardless of purity. Similarly, nickel and yttrium catalyst impurities entrapped within SWCNT and MWCNT were also not found to have a significant role in the toxicity associated with these materials [173]. Ideally, *in vitro* studies utilising metal chelators could shed light on the role of such impurities. However, it must be noted that the conflicting literature might be attributed to the other physico-chemical characteristics of the SWCNT utilised in the individual studies that may have been involved in the observed damage responses. Thus, reiterating the importance of full characterisation of the nanomaterial under study to enable fully informed interpretation.

4.3. Agglomeration (size distribution)

An inherent property of many nanomaterials is their hydrophobicity and thus a propensity to agglomerate particularly under physiological conditions. With regards to human exposure, it is therefore likely that under most circumstances nanomaterials will be in the form of aggregates rather than individual units. Furthermore, given that (geno)toxicity testing, whether *in vivo* or *in vitro* involves dosing in an aqueous carrier or into an aqueous environment (with the exception of dust inhalation studies), the likelihood is that exposure responses will be a consequence of the agglomerated nanomaterial form.

While nanoparticles have a tendency to form larger aggregates, fibrous nanomaterials represent a more complex situation as in addition to aggregation, the fibres themselves may take on a tangled structure depending upon their rigidity, which dramatically alter the dimensions and surface area of the original structure. Fibre rigidity is dependant on the synthesis method, but even the rigid nanomaterials rarely exist as individual units and are attracted to one another by van der Waals forces with a tendency to curve and twist forming bundles [174,175]. Agglomerates are larger and often more rigid than individual nanomaterials, with both SWCNT and MWCNT tending to agglomerate together as wound ropes containing many nanotubes [176].

Various methods are being used to enhance the hydrophilicity of nanomaterials through the use of surfactants or chemical modification (functionalisation) of their surfaces and as a result, the (geno)toxic responses are also altered. For example, some attempts to solubilise CNT through surface modification reported reduced toxicity as a result of the functionalisation [163,177]. In contrast, other studies have found CNT to be more cytotoxic when stabilised with a surfactant, while functionalising nanorods coated with gold and silver also resulted in increased cytotoxicity [178]. Furthermore, some of the agents used to dissolve or functionalise these nanomaterials are of acidic nature or result in oxidation and these have been shown to strongly modify the surface chemistry of the nanomaterials, specifically CNT, and alter their agglomeration behaviour [179,180].

It is worthwhile noting that none of the above-mentioned studies has compared the effects of functionalised or coated nanomaterials to the non-functionalised counterparts, making it difficult to determine if the resultant cellular responses were solely due to the coating. Surface modification of nanomaterials clearly alters their biocompatibility. In some cases it is possible that the coatings themselves are responsible for contributing to the toxic responses directly, or they might function indirectly by enhancing cellular uptake and thus overloading the cellular systems with the nanomaterial, causing the damaging effect. Alternatively, some coatings may shield the cell from the nanomaterial thereby reducing its toxic potential. Additionally, there is no work considering more subtle cellular changes such as genotoxicity, as a consequence of the use of surfactants or functionalisation. However, it is important to remember the scenarios in which human exposure will occur, and although functionalised nanomaterials may be directly used in consumer or clinical applications, the use of surfactants is more difficult to justify as they would not be present in most cases of true physiological exposure situations. Thus, the agglomerated form of the material may be more representative of workplace or consumer exposure scenarios.

Clearly a large amount of effort is needed to establish the interplay between agglomeration, dispersal methods and negative cellular impacts. Furthermore, it is vitally important to fully characterise the level of agglomeration under experimental conditions when performing risk assessment as this could clearly have a dramatic impact upon the interpretation of results. Given that agglomerated structures might no-longer be in the "nano" size range, their exposure associated risks may be substantially reduced perhaps due to reduced cellular uptake or an inability to cross biological barriers. However, currently this information is often missing in studies in the literature and is likely to be a fundamental cause for the lack of consistency and conflicting results often reported.

4.4. Surface charge and chemistry

An understanding of the surface characteristics of nanomaterials under study is critical for providing insight into their behaviour under different experimental conditions. Firstly, surface charge and chemistry will govern the formation of agglomerates according to factors such as the pH or ionic strength of the aqueous environment they are in [181]. Hence, this information can be used to gain insight into the likely aggregation/disaggregation kinetics that may occur during the course of an *in vitro* experiment, or according to the specific biological compartment in which the nanomaterials may become concentrated.

Surface charge also plays an important role in governing cellular uptake of nanomaterials. The plasma membrane is negatively charged (due to the phospholipids on the outer surface), as is the intracellular environment, thus anionic (negatively charged) nanomaterials may be endocytosed at a lower rate than those that are cationic (positively charged). Although this has been observed in practice using PEGylated polylactide and hydrogel nanoparticles of similar sizes but different charges [156,182], this is not always the rule and it does not preclude the uptake of negatively charged nanoparticles [183]. However, nanoparticles with cationic surface charges appear to be associated with greater cytotoxic responses as compared to those with anionic charges, although it is unclear as to whether the cell death is the direct result of the surface charge or if it is because of the increased uptake often associated with cationic nanoparticles [184]. Additionally, DNA is negatively charged, thus cationic nanomaterials may be more likely to interact with the genetic material.

In addition to functionalising the surface of nanomaterials to promote solubility as described in the above section, the surface chemistry may also be modified in order to attach other biomolecules such as peptides for cell targeting or pharmaceuticals for drug delivery. However, these modifications will also have an impact upon the toxicological profile of the resultant nanomaterials. Studies assessing QD with a variety of different coatings demonstrated that cytotoxicity was dramatically influenced by the functionalising group such that organic coatings resulted in the highest toxicity, followed by amine groups, while little impact on cell viability was imparted by carboxyl coatings on the quantum dots [105,185]. Information on surface charge and chemistry is therefore important in explaining uptake mechanisms and predicting likely interactions with biomolecules.

5. The current situation

For many nanomaterials, the evidence accumulating suggests that they may have genotoxic potential. However, there are clearly inconsistencies in the literature that make it very difficult to come to firm conclusions, which are probably due to a number of factors. Currently, there is a lack of consistency in dose metrics used and in the publication of conversion factors. It is therefore impossible to equate units of dose at the moment, which is necessary to facilitate comparisons between studies. Additionally, limited physico-chemical characterisation in most reports means that the true nature of the nanomaterials being investigated (particularly under experimental condition used) is unknown.

An important source of variability is in the selection of test systems. Often only one genotoxicity test is used, which is problematic as most tests measure different endpoints, hence a positive response will be dependent upon the test substances' mechanism of action. For example, the micronucleus assay quantifies clastogenic and aneugenic events resulting in chromosome fragmentation or loss of whole chromosomes (respectively), which lag during cell division and thus become enclosed within a micronucleus rather than the daughter nuclei. On the other hand, the comet assay measures un-repaired DNA strand breaks and alkali-labile sites in DNA, but early apoptotic cells and necrotic cells can skew these results, as they tend to contain fragmented DNA. Often the comet assay measures higher levels of damage than the micronucleus assay because the former will detect breakages that are also repairable. In contrast, the damage measured by the micronucleus assay is that which remains after cell division and thus is more informative of the errors that remain un-repaired and thus have a heavier weighting in the resultant genotoxicity assessment [186].

Bacterial mutagenicity based assays (Ames test), may not be suitable for detecting genotoxicity induced by nanomaterials because these prokaryotes lack the ability to perform endocytosis and the nanomaterials may not be able to diffuse across the bacterial cell wall; this lack of uptake could potentially lead to false negative results. Furthermore, some nanomaterials (such as silver) are antibacterial. There are a small number of reports in the literature that have used the Ames test and found positive (albeit weak) responses for genotoxicity with nanomaterials [116,187], but the applicability of this test needs to be fully investigated if we are to rely on it for nanomaterial risk assessment. Thus, *hprt* or *tk* forward mutation assays utilising mammalian cells may be a more relevant experimental system.

For genotoxicity, there are a number of instances in the literature where studies examining (apparently) the same nanomaterials have reported conflicting results using different assays. For example, both the comet and micronucleus assays indicate that TiO_2 NPs induce DNA damage, whilst the Ames test and chromosome aberration assay report negative results [46,188]. However, there are also some instances where differences between the micronucleus versus the comet assay have been noted within the same publication [73]. Hence, it is clear that a single genotoxicity assay is not sufficient to provide conclusions as to the genotoxic potential of nanomaterials as no single test can cover all potential forms of DNA damage that might arise. The standard regulatory approved way of assessing the genotoxicity of a test substance involves the use of a battery of tests that measure a range of different endpoints, providing information on their mechanism of action. This is also necessary with nanomaterials in order to gain a complete picture of how they interact with cellular systems.

The use of surfactants to disperse nanomaterials also needs careful consideration. Does forced dispersion using a surfactant really reflect potential physiological exposure situations? It can be argued that there are natural surfactants that the nanomaterials may come into contact with (such as the respiratory tract fluid in the lungs). However, this will be largely dependent upon the exposure route. In addition to the use of surfactants generating a false exposure scenario, there is also the possibility that the surfactants may themselves cause a positive result. Thus, surfactants may introduce another degree of complexity that can confound the final results.

A final source of variability may be the cell line utilised for *in vitro* based investigations. Often in many studies cancer cell lines have been the test system of choice, but these may be either more sensitive or more resistant, depending upon their genetic background and stability. Indeed Pacurari and colleagues [149] found that normal mesothelial cells were more susceptible to DNA damage following exposure to SWCNT than malignant mesothelial cells were. Thus, to assess the genotoxic potential of nanomaterials, it is important to ensure that cell lines with suitable stability are utilised to avoid false positive or negative results. There is also a distinct lack of data on the genotoxicity of nanomaterials on germ cells, with only one report to date in the literature that have considered this issue and found ZnO and TiO_2 nanoparticles induce a dose-dependent increase in DNA damage in human sperm cells [189]. This is clearly a very important consideration as recent *in vivo* studies have demonstrated nanoparticle accumulation in the testes following exposure [190,191]. Thus, DNA damage in these cells could have a negative impact on fertility, whilst heritable genetic alterations might result in developmental abnormalities in offspring.

In addition to the above-mentioned confounding factors that introduce a great degree of complexity, there are also a whole host of other aspects that are poorly understood, making it difficult to design and interpret experiments. An issue that requires clarification is the most suitable exposure time to utilise within *in vitro* assays. Traditionally, no more than 24 h exposure periods are used in most genotoxicity testing systems as chemical compounds can readily diffuse into the cells and often their half life also limits their period of activity, thus extended exposures are not necessary. However, it is possible that the uptake and movement of nanomaterials through cells is likely to be slower than chemical diffusion. Furthermore, if these materials are lingering in the cell during mitosis then there is an increased chance that they may interact directly with DNA when the nuclear membrane breaks down, or with components of the mitotic machinery thereby potentially disrupting chromosomal segregation. Thus, longer treatment times may be required before a genotoxic effect is seen upon the test system. Indeed, increased particle uptake with extended incubation time has been observed, such as in the study by Colognato and colleagues [56], who reported that cobalt nanoparticle uptake after 48 h was on average double that of uptake measured at 24 h. Currently however, there are very few investigations that have considered exposures longer than 24 h.

A related issue is the long-term fate of nanomaterials once inside cells, which is another factor that has not been addressed experimentally. It is conceivable that a metallic nanoparticle may itself not induce DNA damage, but if internalised, over time the nanoparticles may corrode to release metallic ions that do induce genotoxicity. Alternatively, if a nanomaterials surface is functionalised to aid its biocompatibility, the stability of those surface coatings over time must be considered and if it does eventually breakdown, then again the genotoxic responses may be significantly different from initial short-term exposure datasets. A 24-h exposure time within an *in vitro* system would be unlikely to detect these effects, leading to false negative results. Thus, long-term fate of nanomaterials once taken up by cells is another important issue that requires attention.

6. Conclusions and recommendations

At present there is limited data on the genotoxic potential of nanomaterials and their possible long-term impact on human health. Most studies have focussed on cytotoxicity and a lot of useful information has been generated relating to the specific aspects of nanomaterial physico-chemical features and their association with toxicity. However, this also needs to be assessed with regards to DNA damage and factors such as cellular accumulation, retention and subsequent consequences need to be considered. The data accumulating in the literature does point to some nanomaterials harbouring the ability to damage DNA, but given the inconsistencies it is difficult to draw conclusions and there needs to be a concerted effort within the scientific community to produce reports that are more informative. Two recent reviews have also addressed this issue and have made recommendations focused around ensuring adequate materials characterisation, use of standardised experimental methods and assessment of the correlation between *in vitro* and *in vivo* results [192,193]. However, there are a number of further considerations; we therefore recommend that investigations should take into account the following:

1. Thorough detailed physico-chemical characterisation (particularly under experimental conditions) is imperative in any nanomaterials' toxicological study. Measurements include size distribution, morphology, surface area, charge, surface modifications, chemical composition (purity), crystallinity and agglomeration. Information on the fabrication process should also be provided.
2. Inclusion of appropriate controls (particle- or fibre-positives and negatives). Additionally, a source of metal ions or the use of metal chelators should be considered in experimental design to establish whether biological effects are the result of nanomaterial interactions, impurities or breakdown products released during exposure.
3. Where functionalised nanomaterials are being assessed, the underivitised form should also be included (if the data is not available elsewhere) to directly determine the influence of the surface modification.
4. Nanomaterial dynamics during the life-span of the (geno)toxicity assay needs to be considered, i.e. what happens to agglomeration and size distribution; if *in vitro* do they settle out or remain in suspension (this might effect adherent versus suspension cells in different ways); do their surface chemistries alter. However, the metrological techniques required to provide answers to many of these questions are currently not available and need to be developed.
5. Consider long-term fate of nanomaterials once internalised (do they corrode to release ionic components, how stable are surface coatings). Ideally, physico-chemical features before and after experimentation should be detailed, but again this may require the development of novel methodologies to provide adequate answers. This information can feed into determining more informative exposure durations for genotoxicity assays.
6. A battery of genotoxicity tests with different endpoints should be utilised to provide insight into their mechanism of action and to ensure that a complete picture of the nanomaterials' reactivity is generated.
7. Consider the genotoxic potential of nanomaterials on germ cells.
8. Extend *in vitro* studies beyond 24 h to determine if longer treatment times are more informative on the genotoxic potential of nanomaterials and consider the modification of other aspects to generate nanomaterial specific test conditions.

Clearly, well designed experiments with consistency in the approaches taken are required to enable a concerted effort to truly determine the hazards associated with nanomaterials, and to define similarities that will enable future extrapolations and predictions. An understanding of the underlying biological mechanisms responsible for the consequences of exposure observed is also required in order to provide information that will allow the informed design of future nanomaterials, ensuring their biocompatibility and minimising potential adverse health risks. This information may prove critical in aiding the progression of the nanotechnology industry, so that it is able to realise the great benefits it promises.

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