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REVIEW ARTICLE

Genotoxic and carcinogenic potential of engineered nanoparticles: an update

Ashutosh Kumar · Alok Dhawan

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Abstract Nanoscience and nanotechnology have seen an exponential growth over the past decade largely due to the unique properties of engineered nanoparticles (ENPs), advances in ENP synthesis, and imaging or analysis tools. The unique properties such as high surface area to volume ratio, abundant reactive sites on the surface, large fraction of atoms located on the exterior face have made these novel materials the most sought after for consumer and industrial applications. This significant increase in the ENP containing consumer products has also enhanced the chances of human and environmental exposure. Humans get exposed to ENPs at various steps of its synthesis (laboratory), manufacture (industry), use (consumer products, devices, medicines, etc.) and through the environment (contaminated water, aerosolized particles, and disposal). Such exposures to ENPs are known to induce genotoxicity, cytotoxicity, and carcinogenicity in biological system. This is attributed to several factors, such as direct interaction of ENPs with the genetic material, indirect damage due to reactive oxygen species generation, release of toxic ions from soluble ENPs, interaction with cytoplasmic/nuclear proteins, binding with mitotic spindle or its components, increased oxidative stress, disturbance of cell cycle checkpoint functions, inhibition of antioxidant defense, and many others. The present review describes an overview of in vitro and in vivo genotoxicity studies with ENPs, advantages and potential problems associated with the methods used in genotoxicity assessment, and the need for appropriate method and approach for risk assessment of ENPs.

Keywords DNA damage · Carcinogenic · In vitro · In vivo · Mechanism of genotoxicity and carcinogenicity · ENPs characterization

A. Kumar (✉) · A. Dhawan
Institute of Life Sciences, School of Science and Technology, Ahmedabad University, Opposite University Bus Stand, University Road, Navrangpura, Ahmedabad 380009, Gujarat, India
e-mail: ashutosh.kumar@ahduni.edu.in

A. Dhawan
Nanomaterial Toxicology Group, CSIR-Indian Institute of Toxicology Research, Mahatma Gandhi Marg, P.O. Box 80, Lucknow 226001, Uttar Pradesh, India

Introduction

Engineered nanoparticles (ENPs) are defined as any intentionally produced particles that have a characteristic dimension between 1 and 100 nm and possess unique physico-chemical properties that are different from their bulk counterparts (Oberdarster et al. 2005). The unique properties such as high surface area to volume ratio, abundant reactive sites on the surface, large fraction of atoms located on the exterior face have made these novel materials the most sought after for consumer and industrial applications (Oberdarster et al. 2005; Stone and Donaldson 2006; Dhawan et al. 2009; Sharma et al. 2012b). ENPs are now increasingly used in plastic wares, clothing, cosmetics, paints, electrical appliances, and even food products (PEN 2013). Their applications also extend into the biomedical field and health care, particularly in medical imaging and diagnosis, wound dressing, pharmaceuticals, drug delivery, and therapy (Nowack and Bucheli 2007). The demand for ENPs in the above-defined market is rising and estimated to reach sales of up to US$1 trillion by 2015 (Roco 2005).

Due to the advances in ENP synthesis, imaging, analysis, and better funding, the development of unique ENPs has opened an opportunity for the growth of novel ways of

rapid disease diagnosis, treatment, and enhancement of the quality of life (Singh 2013). According to the US National Nanotechnology Initiative (NNI), million ton quantities of ENPs (silica, alumina and ceria, ZnO, TiO_2, silver, CNTs, etc.) are being manufactured for various consumer products (USNTC 2004; Kumari et al. 2011). This significant increase in the ENP containing consumer products has also enhanced the chances of human and environmental exposure. Humans get exposed to ENPs at various steps of its synthesis (laboratory), manufacture (industry), use (consumer products, devices, medicines, etc.), and through the environment (contaminated water, aerosolized particles and disposal; Fig. 1; Borm et al. 2006; Hardman 2006; Dhawan and Sharma 2010).

In vitro studies conducted in organotypic cultures and cell lines have shown cytotoxic and genotoxic response of a variety of ENPs synthesized through different methods, with varying chemical compositions, size, shape, surface area, surface coatings, etc. (Osman et al. 2010; Sharma et al. 2009, 2011a, b, 2012a; Shukla et al. 2011a, b, 2013; Vallabani et al. 2011). However, variation observed in the biological responses of ENPs could be due to the differences in the ability to produce the reactive oxygen species (ROS) and their types. Apart from this, the physico-chemical properties (size, shape, surface area, surface coating, composition, dissolution) of ENPs, methods for synthesis, as well as impurities play a major role in their biological outcomes (Dhawan and Sharma 2010). It has also been reported that ENPs interact with the biological macromolecules. Recent studies have shown that ENPs inhibit enzyme activity due to their interaction at the active site or binding directly with the substrate (Kain et al. 2012; Magdolenova et al. 2013). Although several studies have been done for hazard and risk assessment of ENPs, no conclusive data are available regarding their safety. This could be due to the several reasons such as (1) lack of reliable and validated test methods; (2) direct/indirect interference of ENPs with the test methods/reagents; (3) inappropriate characterization of ENPs; (4) methods for ENPs synthesis (Howard 2009; Stone et al. 2009). The lack of consistency in the literature has led to a global effort in formulating a study design that can account for the above confounding factors and consistently predict the mechanism of action, behavior, health risks, and hazards of ENPs.

The Royal Society and Royal Academy of Engineering first raised the concern of risk associated with ENPs exposure (Royal 2004). This prompted several research groups to undertake the cytotoxicity, genotoxicity, and carcinogenicity of ENPs and their products. It is anticipated that due to an exponential increase in the applications of ENPs in various products, there will be a greater need for safety and risk assessment studies in the coming years. Therefore, in the present review, an emphasis has been given to the characteristics of ENPs, currently available methodologies for genotoxicity studies and a survey of the genotoxicological and carcinogenic studies of ENPs and their possible mechanism.

Characteristics of ENPs

The unique physico-chemical (optical, magnetic, electrical) and catalytic properties of ENPs are due to higher surface

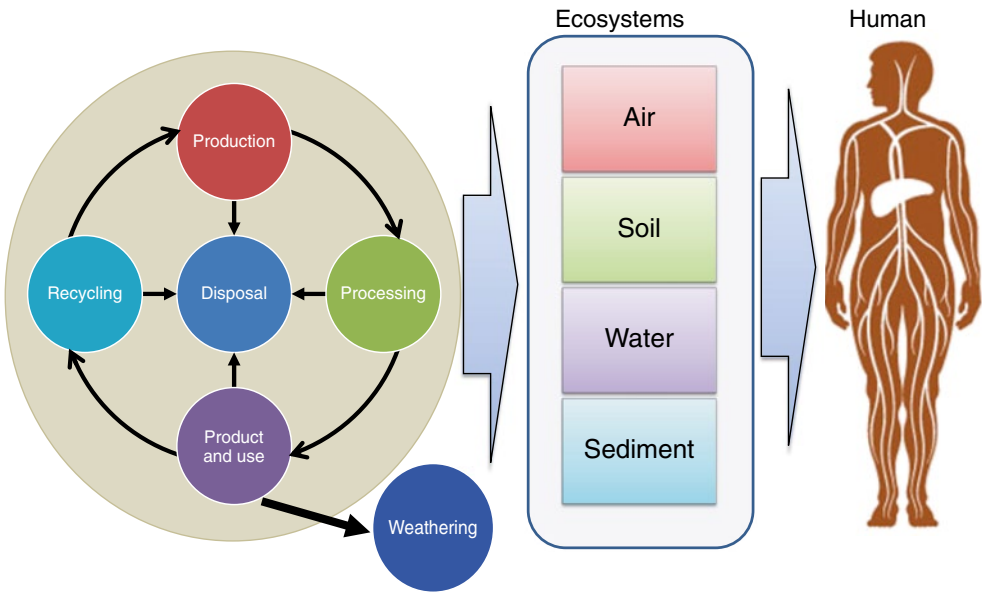

Fig. 1 Life cycle of engineered nanoparticles and exposure paradigm to various ecosystems including human

to volume ratio and an increased number of atoms on particle boundaries than their bulk counterpart (Borm and Berube 2008; Dusinska et al. 2009). These characteristics of ENPs facilitate their diffusion, hardness, dimensionality, formation of suspension, and others.

The optical properties of the ENPs are majorly due to their ability to confine electrons in small size and to produce quantum effects. These structure- and shape-dependent optical absorption properties are exemplified by the change in color of silver suspension from yellow (nanoform of silver) to blue (aggregates of silver). This is also apparent in the case of gold nanoparticles, where the color changes from blue—green—magenta, due to a change in their size and shape.

The ability of the ENPs to form a suspension is also unique. This is due to the higher interaction force between their surface and suspension media which overcome the density differences (Kumar et al. 2012). In case of bulk material, these interactions usually result in a material either sinking or floating in a liquid. ENPs in aqueous suspension are dispersed due to the electrostatic and steric repulsion of the surface charge (positive/negative) present on them (Maynard 2007). Also, the Brownian motion as well as the collision plays an important role in dispersion. As the surface charges of the ENPs skew toward the zero value, the repulsive forces between the ENPs get reduced and ultimately they settle down by gravitational forces. The phenomenon of agglomeration involves the adhesion of particles to each other, mainly because of van der Waal's forces, which dominate at the nanoscale due to the increased surface area to volume ratio (Elsaesser and Howard 2011). Due to agglomeration/aggregation, the physico-chemical properties such as surface charge, size, size distribution, surface to volume ratio, surface reactivity of ENPs get altered thereby modulating their bioavailability and toxicological responses (Navarro et al. 2008; Kumar et al. 2011c).

Diffusion is also a unique characteristic of ENPs as it governs their behavior in the environment. Particle diffusion coefficient is inversely proportional to the particle diameter. As the particle size decreases, diffusional forces become increasingly important and nanoscale particles tend to behave similar to a gas or vapor (Aitken et al. 2004, 2008). Hence, ENPs which have a high diffusion coefficient exhibit high mobility and mix rapidly in an aerosol. After their release in the environment, atmospheric diffusion facilitates the ENPs to migrate speedily from a higher to a lower concentration, thus resulting in rapid dispersion and potential for particles to travel a great distance from the source increasing the risk of environmental impact (Feliu and Fadeel 2010).

Other special properties of ENPs are the quantum confinement in semiconductors, surface plasmon resonance in some metal particles, and superpara-magnetism in magnetic materials. For example, ferroelectric materials smaller than 10 nm can switch their magnetization direction using room temperature thermal energy, thus making them unsuitable for memory storage. Also, copper nanoparticles smaller than 50 nm are considered super hard materials that do not exhibit the same malleability and ductility as bulk copper (Science Daily 2013).

Considering the unique properties that the ENPs attain, it is prudent that the particles should be thoroughly characterized for their physico-chemical properties before assessing the impact on human and environmental health. It has been proposed that the properties to be characterized should include the following: particle number, mass concentration, surface area, porosity, roughness, morphology, surface charge, surface chemistry, reactivity, size, size distribution, aggregation, elemental composition, structure, shape, purity, crystallinity, solubility, etc. determination of the hydrodynamic size, size distribution, zeta potential, dispersity, and the concentration and time at which agglomeration occurs should also be performed for better understanding of behavior of ENPs and interpretation of results.

Methods used for genotoxicity assessment

The genotoxicity of the ENPs can be assessed using prokaryotic system, cell culture in vitro and in vivo models. Initial screening for genotoxicity is done using the bacterial reverse mutation assay (Ames test). Subsequently, the ENPs are assessed for their ability to induce various kinds of DNA damage in cultured mammalian cells (either cell lines or primary cultures) using different assays such as the comet assay, gene mutation assays [hypoxanthine phosphoribosyl transferase (*HPRT*), thymidine kinase (*TK*), phosphatidylinositol glycan, Class A (*Pig-a*), and others], micronucleus assay, and chromosomal aberration test (Kumar et al. 2013). The genotoxic potential of ENPs is finally confirmed using in vivo studies.

The Ames test is a bacterial mutation assay based on the reversion of histidine auxotrophs to autotrophs. Bacterial strains mutated at histidine locus do not synthesize histidine and thus die when plated on an agar medium lacking histidine (Ames et al. 1975; Mortelmans and Zeiger 2000). However, reversal of mutation in histidine gene induced by some compound/ENPs will enable the bacterium to synthesize histidine and form a visible colony in minimal histidine medium. The structure of the bacterial cell wall is rigid and semipermeable. It does not allow the larger ENPs to cross the cell wall. Hence, to increase the appropriateness of the Ames test for ENPs, the tester strains have been modified with deep rough (rfa) mutation, which eliminates the polysaccharide side chains of lipopolysaccharides, making the bacteria more permeable. The Ames test has been widely used to assess the genotoxicity of a variety of

NPs (Di Sotto et al. 2009; Maenosono et al. 2007, 2009; Yoshida et al. 2009; Kumar et al. 2011a, b).

The hypoxanthine–guanine phosphoribosyltransferase (HPRT) forward mutation assay is an in vitro mammalian cell gene mutation test used for the assessment of the genotoxicity of a substance (Finette et al. 2002). V79 Chinese hamster cells are used in this assay, which have one functional copy of the HPRT gene located on X-chromosome which helps in the phosphoribosylation of hypoxanthine and guanine. During the test procedure, cells are grown in the presence of the toxic analog of guanine, i.e., 6-thioguanine. The HPRT enzyme acts on this analog and enables the incorporation of poisonous 6-thioguanine into DNA during replication which leads to the cell death. However, if any mutation (spontaneous and induced) occurs in the HPRT gene, the salvage pathway does not function properly, and hence, the toxic 6-thioguanine will not be incorporated during the DNA synthesis. Therefore, the colony survival frequency represents the frequency of deleterious point mutations of the test substance at a given dose. This assay has been used to assess the genotoxicity of the ENPs showing largely negative results (Wang et al. 2007, 2011).

Comet assay is a simple, rapid, and sensitive technique used to detect the single- and double-stranded DNA break in individual cells (in vitro and in vivo; Bajpayee et al. 2013; Kumar et al. 2013). This is the most frequently used screening test for the quantification of alkali-labile sites, oxidative DNA damage, DNA–DNA or DNA–protein cross-links and abasic sites. Single-cell suspension in low melting point agarose is spread onto a normal melting agarose pre-coated microscope slide to make a monolayer of cells. The cells are then lysed in high salt concentration to unwind the super-coiled DNA and reveal the damage. Further, electrophoresis is performed to stretch out the fragmented DNA based on molecular weight. The intact DNA, due to its large size migrates minimally, whereas the fragmented DNA moves further toward the anode. The migration of DNA is detected using different stains (ethidium bromide, YoYo-1, silver nitrate, sybr green, etc.). When visualized under the microscope, the migrated DNA along with the undamaged DNA forms a comet-shaped structure which is analyzed for quantitative and qualitative parameters using commercially available softwares (Comet Assay Forum 2013). Comet assay has been used to detect the damaged bases by incubating nucleoids with lesion-specific endonucleases, such as endonuclease III (Endo III) and formamidopyrimidine DNA glycosylase (FPG) that recognize oxidized pyrimidines and purines, respectively (Collins et al. 1996; Dusinska and Collins 1996). Comet assay has been widely used for the assessment of the genotoxic potential of ENPs (Karlsson et al. 2004; Stone et al. 2009; Karlsson 2010; Sharma et al. 2009, 2012a; Shukla et al. 2011b, 2013).

However, it has been shown that the presence of ENPs in the comet head (nucleoid) interferes with the assay by inducing the additional DNA damage (Karlsson et al. 2004; Stone et al. 2009; Karlsson 2010). It has also been shown that the ENPs and ions released due to dissolution of the particles interact with FPG enzyme leading to an inhibition of enzyme activity which hampers the detection of oxidatively damaged DNA in the comet assay (Kain et al. 2012). This inhibition could be attributed to the binding of ions to the –SH groups at the active site or due to physical hindrance by ENPs.

Micronucleus is a chromatin-containing structure in the cytoplasm surrounded by a membrane without any detectable link to the nucleus. They are formed during the anaphase stage of cell division from the lagging chromosomes or their fragments. The micronucleus test is based on the scoring and comparison of the micronucleus in control and treated cells. In the modified micronucleus assay, a cytokinesis blocking agent (cytochalasin-B) is added to inhibit cell division which gives binucleated appearance to the cells. This enables a more accurate scoring by reducing the incidence of false positives. This method is easier and faster to perform than the chromosomal aberration test and has a better potential for automation. This assay is widely used to assess the genotoxic and carcinogenic potential of the ENPs (Falck et al. 2009; Sharma et al. 2009; Di Virgilio et al. 2010; Shukla et al. 2013). However, at higher concentrations, the deposition of ENPs on the cell surfaces during treatment/slide preparation hinders the counting of micronucleus (Falck et al. 2009; Di Virgilio et al. 2010; Shukla et al. 2013).

A more specific tool to detect the double-strand breaks is the analysis of *γ-H$_2$AX*. This is one of the components of nucleosome core histone H_2A family. The phosphorylation of this protein at serine-139 is mediated either by ataxia telangiectasia mutated (*ATM*), ataxia telangiectasia, and Rad3-related protein (*ATR*) or DNA-dependent protein kinase (*DNA-PK*) leading to the formation of *γ-H$_2$AX*. *γ-H$_2$AX* is present in a complex form in the cell, and DNA double-strand breaks (DSB) trigger its phosphorylation. This converts the complexes into monomers which are thought to act as signals to recruit and retain DNA repair proteins to the DSB site. The alteration in the expression profile of γ-H_2AX induced by ENPs has been detected by different techniques such as immunohistochemistry, flow cytometry, and Western blot (Ismail et al. 2007; Lewis et al. 2010).

ENPs and their genotoxic potential

ENPs are frequently used in the commercial and industrial products. They are used as pigments to enhance the

appearance, imaging agent, and drug carrier in biomedical applications, in antibacterial agents, and in consumer products to improve the durability. Various studies have been performed with engineered nanoparticles, and an overview of the in vitro and in vivo genotoxicity studies with ENPs is summarized in Tables 1 and 2, respectively.

ENP-induced genotoxicity can be attributed to several factors, such as direct interaction of ENPs with the genetic material, indirect damage due to ROS generation, release of toxic ions from soluble ENPs (Kisin et al. 2007; Barnes et al. 2008). Other factors such as interaction of ENPs with cytoplasmic/nuclear proteins, binding with mitotic spindle or its components, increased oxidative stress, disturbance of cell cycle checkpoint functions, generation of ROS at the ENP surface or by interaction with cellular components, inhibition of antioxidant defense are also reported to induce genotoxicity (Fig. 2; Dhawan et al. 2009; Dhawan and Sharma 2010).

Due to the size of the ENPs, the probability of their internalization into the cells and interaction with cellular organelles and macromolecules (DNA, RNA, and proteins) is very high. These interactions can damage the genetic material and cellular organelles by physical injury as well as by modulating the biochemical pathways. Using in silico approaches, it has been shown that carbon ENPs bind to single-stranded DNA and get incorporated into DNA duplex structures during the DNA replication (An et al. 2010). This also suggests that carbon ENPs may interfere in DNA replication process (An et al. 2010). Also, the strong interaction of other ENPs with the DNA and DNA bases in different organisms has been reported (An et al. 2010; Jin et al. 2012).

Additionally, ENPs have the potential to interact with the proteins involved in the essential cellular pathways such as DNA replication, transcription and repair; mitotic spindle apparatus, centrioles and their associated proteins. The binding efficiencies of ENPs with different essential proteins have been investigated by some in silico and in vitro studies. An in silico study by Baweja et al. (2011) showed that C60 fullerene interacts at the ATP binding domain of human DNA topoisomerase II alpha and could inhibit the enzyme activity (Baweja et al. 2011). Another in silico study showed that C60 fullerene might interact with PMS2, RFC3, and PCNA proteins involved in the DNA mismatch repair pathway (Gupta et al. 2011). It has been proposed that ENPs bind to the active site of the protein leading to their structural/conformational changes. This could also result in the competitive inhibition of the enzyme due to the inability of the substrate to bind (Huang et al. 2009; Jugan et al. 2012; Kain et al. 2012). Jugan et al. (2012) have shown that titanium dioxide nanoparticles exhibit genotoxicity and impair DNA repair activity in A549 cells. The inactivation of the DNA repair protein activity has been attributed to the ROS generation (Jugan et al. 2012).

ENPs of various kinds are reported to induce ROS and oxidative stress under in vitro and in vivo conditions (Karlsson et al. 2009; Xie et al. 2010; Heng et al. 2011a, b; Khan et al. 2011; Landsiedel et al. 2012; Sharma et al. 2012c; Shukla et al. 2011b, 2013). Low concentrations of ROS can activate the signaling pathways (Mates et al. 2012). However, at higher concentrations, ROS induces lipid peroxidation, damage to mitochondria, macromolecules, and cell membrane. Mitochondria are the major source of the oxygen-free radicals and also a major target of ROS-induced oxidative stress and damage. Under oxidative stress, mitochondria release various pro-apoptotic factors due to an increased permeabilization of outer membrane and the depolarization of the inter-membrane potential (Fig. 3; Cadenas and Davies 2000).

ROS can directly attack the DNA and can generate various modified DNA bases. Among these bases, 8-oxo-7,8-dihydroguanine (8-oxoG) is the most abundant and seems to play a major role in mutagenesis and carcinogenesis. It has been shown that the levels of 8-oxoG can be used as an indicator of oxidative DNA damage after the exposure of ENPs using the FPG-modified comet assay (Kim et al. 2011; Asare et al. 2012; Magdolenova et al. 2013) Also, the level of 8-oxoguanine DNA glycosylase (OGG1), which is involved in base excision repair of 8-oxoG, is found to be induced by ROS (Hudecova et al. 2012). It has been shown that in the liver of rats treated with C60 fullerene, there is an increased mRNA expression of OGG1; however, a corresponding increase in its repair activity is not observed (Foldbjerg et al. 2012). It has also been observed that pre-treatment with the free radical scavenger *N*-acetyl-L-cysteine (NAC) can inhibit the genotoxicity induced by ENPs (Guo et al. 2011; Sharma et al. 2012a). This helps in understanding the mechanism of ROS-induced cellular perturbation including DNA damage and apoptosis.

ENPs and their carcinogenic potential

In vitro and in vivo studies have revealed that ENPs induce DNA damage and mutations. The association between genotoxicity and cancer is also well-known. Hence, these studies provide invaluable information in predicting the carcinogenicity of ENPs. For example, the carcinogenic effects of ionizing radiation, UV radiation, and many chemical carcinogens are due to their ability to cause DNA damage and gene mutations. The correlation between the metals, metal oxides, oxidative stress, and cancer has been extensively reviewed (Banin et al. 1998; Barchowsky and O'Hara 2003; Pulido and Parrish 2003; Valko et al. 2005; Lee et al. 2012). It is well accepted that excessive generation of ROS, overwhelms the antioxidant defense mechanism of the cells through oxidation of biomolecules. The

Table 1 Selected in vitro genotoxicity studies of ENPs

ENPs type	Phase/size	Genotoxicity test	Result	References
Titanium dioxide (TiO_2)	Anatase 10, 20, 200, >200 nm and rutile, 200 nm	FPG-modified comet assay, CBMN test	Photocatalytic activity of the anatase particle was higher than that of the rutile form. Rutile particles induced oxidative DNA damage in the absence of light but the anatase particle of 200 nm size did not	Gurr et al. (2005)
	Ultrafine particles with organic and inorganic coatings 14–60 nm	Chromosomal aberration	No increase in chromosomal damage frequencies observed in the presence or absence of UV	Theogaraj et al. (2007)
	100 nm	CBMN test, comet assay, and HPRT gene mutation assay	130 μg/ml treatment increases the MNBC frequency by two to threefolds and mutation frequency by 2.5 fold. 65 μg/ml treatments induce fivefold increases in comet tail moments	Wang et al. (2007)
	25 nm	CBMN test, Comet assay	Induces micronucleus frequencies, ROS generation and activation of p53-mediated DNA damage checkpoint signals	Kang et al. (2008)
	Degussa P25, 24.4 ± 0.5 nm	FPG-modified Comet assay and CBMN test	Particles with UVA irradiation induce DNA strand breaks (including oxidative damage to the DNA)	Vevers and Jha (2008)
	40 nm rutile, <25 nm anatase and 5 μm bulk	Comet assay	Nanosized anatase are more effective than the rutile, but none of them were a potent inducer of DNA damage	Falck et al. (2009)
	Anatase	Comet assay	Significant increase in DNA damage at all concentrations	Gopalan et al. (2009)
	Anatase, 5 and 40 nm	HPRT gene mutation assay	TiO_2 NPs are internalized by the cells and induced the mutation frequency in MEF cells	Xu et al. (2009)
	Anatase <25 nm, rutile <100 nm	FPG- and Endo III-modified comet assay and expression studies on DNA damaging responsive gene in real-time quantitative PCR	Anatase particles are potent to induce DNA damage than rutile. Whereas, rutile form are strong inducer of oxidative stress related genes as compared to anatase	Petkovic et al. (2011)
	20 ± 7 nm	MN test SCE assay	MN frequencies increase at 0.5 and 1 μg/ml; however, SCE increased at 1–5 μg/ml concentrations	Di Virgilio et al. (2010)
	Anatase 50 nm	FPG-modified comet assay and CBMN test	A significant increase in oxidative DNA damage and micronucleus frequencies was observed in A431 cells exposed to concentrations 0.8, 8 and 80 μg/ml	Shukla et al. (2011b)

Table 1 continued

ENPs type	Phase/size	Genotoxicity test	Result	References
	Anatase, oval shape, length 76 ± 41 nm, width 53 ± 22 nm	Comet assay	No increase in DNA damage	Hackenberg et al. (2011a)
	Anatase, 50 nm	FPG-modified comet assay and CBMN test	Oxidative DNA damage and the frequency of MN formation increased in a dose-dependent manner	Shukla et al. (2013)
	Anatase <100 nm <100 nm	Electron paramagnetic resonance (EPR) 8-OHdG analysis	TiO_2 treatment showed no DNA breakage, DNA neither adduct nor free radical generation	Bhattacharya et al. (2009)
	140 ± 44 nm, 79 % rutile and 21 % anatase	Ames test and Chromosomal aberration (CHA)	Negative results in both the tests	Warheit et al. (2007)
	63 nm	Comet assay Comet assay (FPG)	Observed significant increase in DNA damage	Karlsson et al. (2008a)
		Micronucleus (MN) test sister chromatid exchange (SCE)	MN frequencies as well as SCE frequencies increased in dose-dependent manner in exposed cells	Turkez and Geyikoglu (2007)
		Comet assay	Dose-dependent increase in DNA damage induced and no oxidative lesions were measured	(Karlsson et al. 2008b)
Zinc oxide (ZnO)	100 nm	Chromosomal aberration assay	NPs were found to be clastogenic under all conditions: simultaneous irradiation > pre irradiation > dark	Dufour et al. (2006)
	30 nm	Comet assay	Significant DNA damage was observed in dose-dependent manner	Sharma et al. (2009)
	10 and 20 nm	Comet assay with/without FPG enzyme	Significant DNA damage was observed in NPs exposed cells with and without FPG	Gerloff et al. (2009)
	19.6 ± 5.8 nm	Comet assay	Significant genotoxic effect was observed at 5 and 10 μg/ml	Yang et al. (2009)
	70 ± 13 and 420 ± 269-nm	Comet assay	Significant DNA damaging potential was observed at 10, 12 and 14 μg/ml of concentration	
		Standard comet assay (Alkaline)	Significant DNA damage was observed in both cells exposed to ZnO NPs	Gopalan et al. (2009)
	30 nm	Comet assay and CBMN test	Concentration- and time-dependent increase in DNA and cytogenetic damage were observed in exposed cells with increasing nanoparticle concentrations	Osman et al. (2010)
	30 nm	Comet assay with/without FPG	Significant DNA damage in comet assay was observed	Sharma et al. (2011b)
	30 nm	Comet assay	A significant increase in DNA damage was observed in exposed cells	Sharma et al. (2011a)

Table 1 continued

ENPs type	Phase/size	Genotoxicity test	Result	References
	Oval shape, length 76 ± 41 nm, width 53 ± 22 nm	Comet assay	Cumulative genotoxic effects of ZnO NPs were demonstrated after 24-h exposure at sub-cytotoxic concentrations in human nasal mucosa cells	Hackenberg et al. (2011c)
	71 nm	Comet assay with/without FPG	Significant DNA damage in comet assay was observed	Karlsson et al. (2008a)
Iron oxide	<70 nm	Comet assay	No genotoxicity was observed in exposed cells	Auffan et al. (2006)
	29 nm, Fe_2O_3	Alkaline and FPG-modified comet assay	No significant DNA damage was observed at any concentration	Karlsson et al. (2008b)
	20–30 nm, Fe_3O_4	Alkaline and FPG-modified comet assay	Oxidative DNA damage was found only at 80 μg/ml of Fe_3O_4 NPs	Karlsson et al. (2008a)
	30–60 nm, Fe_2O_3	Alkaline and FPG-modified comet assay	NPs did not induce significant DNA damage while micro particles induced DNA damage at 80 μg/ml, whereas oxidative DNA damage was not observed	Karlsson et al. (2009)
	20–40 nm, Fe_3O_4	Alkaline and FPG-modified comet assay	DNA damage was higher in micro particles exposed cells than the NPs treated cells. Whereas, oxidative stress was higher in NPs treated cells as compared to micro particles treated cells	Karlsson et al. (2009)
	50 nm	Alkaline comet assay and detection of 8-OH-dG using ELISA method	Significant DNA damage was found at 50 and 250 μg/ml in IMR-90 cells, whereas in BEAS-B2 cells, it was on 250 μg/ml. However, no significant increase in the level of 8-OH-dG was observed	Bhattacharya et al. (2009)
	311 nm	Comet assay and CBMN assay	All magnetite fractions induced DNA damage in a concentration-dependent manner after 4-h exposure. However, the MN frequencies were significantly after 24-h exposure	Konczol et al. (2011)
	3 nm, FePt NPs capped with 2-aminoethanethiol (AET)	Ames test	No mutagenicity was observed in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537), and *Escherichia coli* WP2uvrA	Maenosono et al. (2009)
	10 nm—uncoated Fe_2O_3 NPs, Dextran coated Fe_2O_3 NPs, Uncoated Fe_3O_4 NPs, Dextran coated Fe_3O_4 NPs	CBMN	Only dextran coated Fe2O3 has shown the increase in the number of CBMN	Singh et al. (2009)
Cerium oxide	7 and 320 nm	Comet assay	Significant DNA damage was observed at 60 μg/ml and above concentration. Nano ceria is also found to be more genotoxic than their bulk counterpart	Auffan et al. (2009)

Table 1 continued

ENPs type	Phase/size	Genotoxicity test	Result	References
	5–20 nm	8-OH-dG using Oxy DNA assay kit	Significant increased the level of 8 OH-dG; was observed after 20 and 30 min exposure	Rothen-Rutishauser et al. (2009)
	5.4 nm	Comet assay and SCE assay	No significant DNA damage and SCE was observed in exposed cells	Pierscionek et al. (2010)
	3–5 nm	Comet assay	No significant DNA damage was observed	Das et al. (2012)
Aluminum oxide	<50 nm	Alkaline comet assay	Significant DNA damage was found in L5178Y cells at all concentrations of NPs with S9, while without S9, it was only at higher concentration. All the concentrations of NPs with and without S9 have shown DNA damage to BEAS-2B cells	Kim et al. (2010)
	0.2 nm	CBMN assay gamma-H2AX immuno staining cytogenetic analysis (FISH)	Al_2O_3 NPs induced significant increases in micronucleus frequency as compared to control. It also induced the incidences of chromosome loss and polyploidy in samples treated with 2 mg/T-75 of Al_2O_3 nanoparticles	Tsaousi et al. (2010)
	28 nm	Micronucleus (MN) test Sister chromatid exchange (SCE)	Increase in MN frequencies was observed at 0.5–10 μg/ml concentration. However, SCE was observed at 1–25 μg/ml	Di Virgilio et al. (2010)
Copper oxide	42 nm	Alkaline and FPG-modified comet assay	Concentration-dependent increase in oxidative DNA damage was observed	Karlsson et al. (2008a)
	20–40 nm	Alkaline and FPG-modified comet assay	Induction of FPG lesions (oxidative DNA damage) were observed in NPs exposed cells, whereas, no significant FPG lesions were observed in macro particles exposed cells	Karlsson et al. (2008a)
	28 nm	Alkaline comet assay	DNA damage was observed in nanoparticles exposed cells	Midander et al. (2009)
Silver	30 nm	Chromosomal aberration and aneuploidy	Dose-dependent chromosomal aberration and aneuploidy was observed in the treated Medaka fish cell line	Wise et al. (2010)
	6–20 nm	Comet assay, CBMN	DNA aberrations were more prominent in cancer cells with more chromosomal aberrations	Asha Rani et al. (2009)
	25 nm polysaccharide surface functionalized and uncoated nanosphere	Immunoblot analysis of DNA repair pathway genes	Upregulation of p53, Rad 51 and phosphorylated H2AX protein expression. Coated AgNP show more severe damage than uncoated AgNP	Ahamed et al. (2008)

Table 1 continued

ENPs type	Phase/size	Genotoxicity test	Result	References
	46 ± 21 nm	Comet assay Chromosomal aberration	DNA damage and chromosomal aberrations were observed at ≥0.1 μg/ml concentration	Hackenberg et al. (2011b)
	5–10 nm	Comet assay	DNA damage were observed at concentration more than 0.2 mg/l	Eom and Choi (2010)
	40–60 nm	Comet assay	DNA damage were observed at concentration 50 and 100 μg/ml after 5 min and 3 h of exposure	Flower et al. (2012)
Carbon	C_{60} (polyhydroxylated)	CBMN	No genotoxicity were observed at all doses (11–221 μM)	Mrdanović et al. (2009)
	C_{60}	Ames test	No mutagenicity were observed	Shinohara et al. (2009)
	SWCNT-MWCNT	CBMN and Sister Chromatin	No genotoxicity effects were observed but SWCNT induces mitotic inhibition	Szendi and Varga (2008)
	MWCNT	Chromosome aberration test and Ames test	No mutagenic and clastogenic effects were observed	Wirnitzer et al. (2009)
	MWCNT	Ames test	No mutagenicity were observed	Di Sotto et al. (2009)
	SWCNT diameter 8 nm; length <5 μm	Comet assay	DNA damage was observed at concentration 5 and 10 μg/ml after 24 h of treatment	Yang et al. (2009)
	MWCNT	Comet assay with and without FPG	Dose-dependent increase in the DNA damage was observed at concentration 1, 20, 40 μg/ml, after 4 h of treatment	Karlsson et al. (2008a)
	MWCNT diameter 5–20 nm; length 401.3 nm	CBMN	Dose-dependent increase in the number of micronuclei were observed at treatment concentration 10–50 μg/ml after 24 h	Srivastava et al. (2011)
	178.6 nm C_{60}	Comet assay	Dose-dependent increase in the DNA damage was observed at treatment concentration 0.022–110 μg/l, after 3 and 6 h of treatment	Dhawan et al. (2006)
	C_{60}	gpt delta mutagenicity assay	Mutagenicity were observed in a dose-dependent manner at concentration 0.1–30 μg/ml after 3 days exposure	Xu et al. (2009)

Table 2 Selected in vivo genotoxicity studies of ENPs

	Phase/size	Genotoxicity test	Result	References
C_{60} fullerenes		Bone marrow micronucleus test on ICR mice	No in vivoclastogenic ability of C_{60} were observed up to 88 mg/ml	Shinohara et al. (2009)
Single-walled carbon nanotubes (SWCNT)		Comet assay	DNA damaging potential of SWCNT was observed	Jacobsen et al. (2009)
Multi walled carbon nanotube (MWCNT)	Diameter 11.3 nm and length 0.7 μm	Micronucleus assay	A significant increase in the micronuclei was observed after 3 days intratracheal administration at concentration 0.5–2 mg/rat	Muller et al. (2008)
C_{60} and single-walled Carbon nanotubes (SWCNT)		8-OHdG analysis	Both NPs were associated with increase in 8-OHdG in liver and lungs at doses of 0.064 and 0.64 mg/kg of body weight, respectively	Folkmann et al. (2009)
TiO_2	25 nm	8-OHdG analysis	No change were observed in female Wistar rats at exposure concentration up to 1.2 mg/lung	Rehn et al. (2003)
TiO_2	Anatase/rutile, 21 nm	Comet assay MN test gamma-H2AX immunostaining 8-OHdG analysis	Increase was observed in 8-OHdG and gamma-H2AX foci. The numbers of MN were also increased at concentrations 60, 120, 300, 600 μg/ml treatments	Trouiller et al. (2009)
Ag	60 nm	Bone marrow micronucleus test in Sprague–Dawley rats	No significant increase in (micronucleated erythrocytes) was observed up to dose 1,000 mg/kg body weight	Kim et al. (2008)
Silica	37 and 83 nm		No significant pulmonary, inflammatory, genotoxic or adverse lung histopathological effects were observed at the exposure exposed to 3.7×10^7 and 1.8×10^8 particles/cm^3	Sayes et al. (2010)
Magnetite	9.4 nm	Micronucleus assay	Significant increases in the micronuclei were observed after 24 h of intraperitoneal treatment of magnetic fluid containing 5×10^{15} and 5×10^{17} particles/kg	Freitasa et al. (2002)
Magnetite nanoparticles surface coated with polyaspartic acid	8.5 nm	Micronucleus assay	Increase in the MN frequency was observed in bone marrow cells of Swiss mice	Sadeghiani et al. (2005)
ZnO	50 nm	Micronucleus assay	No micronucleus was observed at the concentration up to 5 g/kg body weight	Li et al. (2011)

Table 2 continued

	Phase/size	Genotoxicity test	Result	References
ZnO	30 nm	Comet assay with and without FPG	Significant increase in the oxidative DNA damage was observed in Swiss mice liver cells at dose 300 mg/kg body weight	Sharma et al. (2012c)
Au	2, 20 and 200 nm	Micronucleus assay	No micronucleus was observed in male Wistar rat after 3 days exposure of 18 μg/lung	Schulz et al. (2012)
CdSe QDs	5.1 nm	Micronucleus assay	Significant increase in the micronucleus was observed in bone marrow cells of albino mice at 500, 1,000, and 2,000 mg/kg body weight	Khalil et al. (2011)

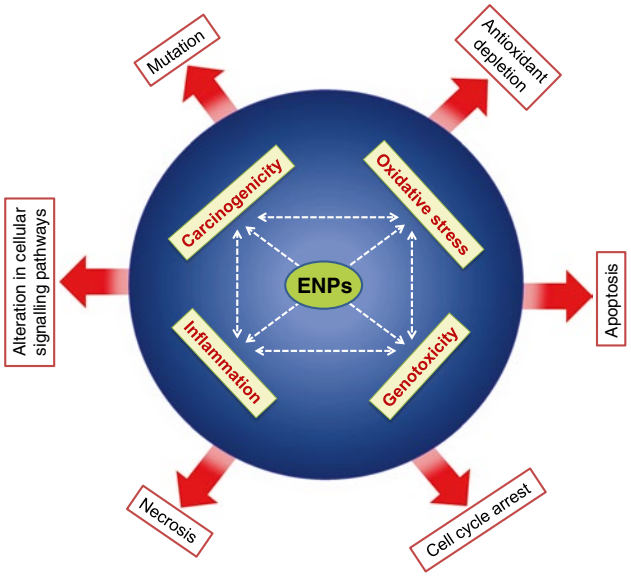

Fig. 2 Cellular effects of engineered nanoparticles

role of oxygen-derived species in causing cell injury or death is increasingly recognized: ROS is involved in a large number of degenerative changes, leading to tissue degradation, a hallmark in carcinogenesis, aging, and other diseases (Luo et al. 2011). It also compromises the immune system leading to an increased microbial load resulting in cell and tissue damage. It is now well established that free radicals produce different types of genetic damage which could lead to cancer. Among oxidative DNA damage products, 8-OHdG is the most studied due to its relative ease of measurement and premutagenic potential. Elevated 8-OHdG has also been reported in numerous tumors, strongly implicating such damage in the etiology of cancer (Oberley 2002). Several studies have shown that the ENPs have capability to induce the level of 8-OHdG in different cell models suggesting the carcinogenic potential.

ENPs can induce oxidative stress and subsequently can elicit inflammatory responses, which could act as an initiator of carcinogenesis. ENPs are highly reactive because of the presence of electrons on their boundary. They are also more likely to adsorb endogenous substances, react with proteins and enzymes, and trigger cytokine release. This could mediate inflammatory responses and potentially initiate a series of toxic responses far from the initial site of deposition (Borm and Kreyling 2004; Bergamaschi et al. 2006). C60 fullerene, for example, was reported to cause photo-induced DNA damage by interacting with NADH, which is an endogenously present reducing agent (Wang et al. 2009). Similarly, carbon nanotube exposure has been associated with adverse cardiovascular effects by causing aortic DNA damage, platelet aggregation, and enhanced vascular thrombosis through inflammatory events

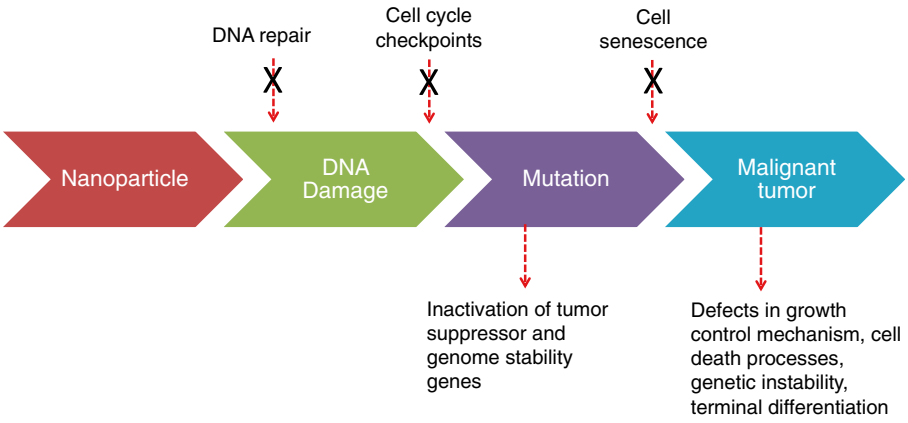

Fig. 3 Schematic showing the involvement of cellular processes in ENP-induced genotoxicity and carcinogenicity

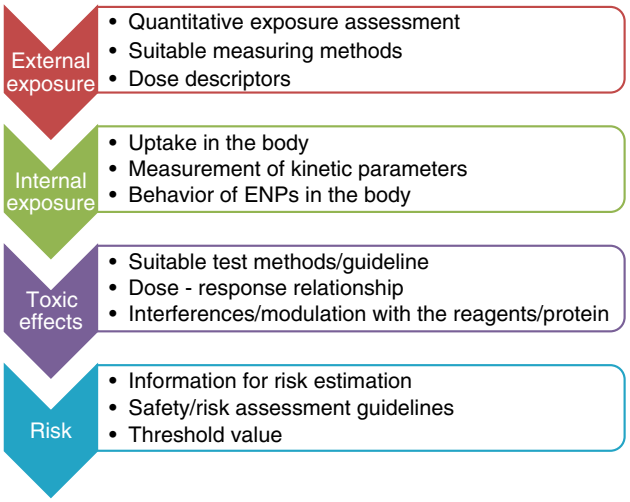

Fig. 4 Knowledge gaps in human risk assessment of engineered nanoparticles

(Radomski et al. 2005; Ng et al. 2010). TiO_2 ENPs are reported to induce inflammatory cytokines and apoptosis in cell lines derived from different organs as well as in vivo (Petkovic et al. 2011; Shukla et al. 2011a, 2013).

The deposition and translocation of ENPs in the cellular systems are also a big concern. Studies have demonstrated that ENPs are not only deposited at the site of injection but it can get deposited away from the site of injection (Takagi et al. 2008; Sakamoto et al. 2009). This suggests that ENPs may easily translocate from one organ to other and also exert effects away from organ of exposure. It is also true that the deposition and translocation property of ENPs are dependent on their size, shape, and physico-chemical properties. Earlier reports from Takagi et al. and Sakamoto et al. have demonstrated the carcinogenic potential of MWCNTs (Takagi et al. 2008; Sakamoto et al. 2009), whereas Muller et al. conducted similar tests with MWCNTs and reported no carcinogenicity after a 2-year period of exposure (Muller et al. 2009). The difference in the results may be due to the variation in the size and length of the nanotube as well as the model used for the study. Takagi et al. have used p53 knockout mice for their study which could be more sensitive to carcinogenic reaction. Such similar studies and the contradictory findings not only provide some insight about the outcome of ENPs toxicity but also underscore the importance of the process used to synthesize the ENPs as well as the study design/protocols used during experimentation.

Biopersistence of ENPs also poses a certain degree of adverse health effect to human and environment. As the ENPs size is very small, the likelihood for their entry into the cell as well their persistence into the cell is also higher. For instance, when the clearance rate of ENPs is slower than the accumulative rate, the presence of ENPs will remain in the organ. Additionally, the exposure and persistence of those ENPs, which can induce the mutation, will increase the risk of developing cancer. To address this concern, the mutagenic potential of the ENPs was assessed by bacterial reverse mutation assay (Sera et al. 1996; Yoshida et al. 2009; Kumar et al. 2011a, b, d, e) and extrapolated with the carcinogenic properties. Kumar et al. have demonstrated the frame shift mutagenic potential of the ZnO and TiO_2 ENPs in Ames test (Kumar et al. 2011b), whereas Sera et al. demonstrated the potential of C60 fullerene ENPs to exert mutagenic activity due to the oxidized phospholipids in rat liver microsomes (Sera et al. 1996).

Based on the epidemiological studies conducted among the male production workers at TiO_2 industry from Western Europe and North America, it was predicted that the workers were on high risk for lung and kidney cancer with comparison to general population (Ng et al. 2010). However, the data were not enough to conclude the association between occupational exposure of TiO_2 ENPs and cancer risk. In contrast, sufficient in vitro reports are available to exhibit the genotoxic potential (such as micronucleus formation, DNA damage) of TiO_2 ENPs. Several in vivo experiments

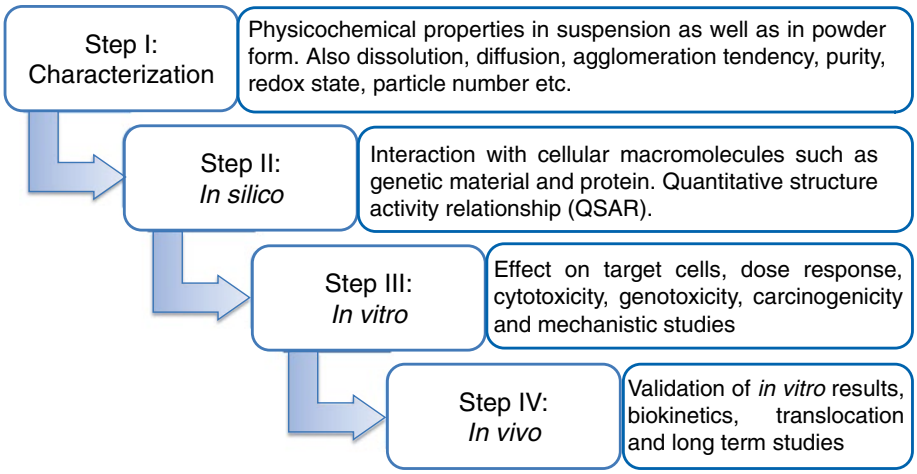

Fig. 5 Approaches in nanomaterial toxicity testing

also demonstrated that exposure of TiO_2 ENPs increases the probability for the tumor incidence in the experimental animals. The shortcoming with these tests and reports is the short-term treatment of ENPs. It is suggested to perform the in vitro and in vivo genotoxicity testing for longer periods to observe whether there are long-term effects of ENPs such as tumor formation and carcinogenesis. It will also be useful to look at the clearance of ENPs from the body and to study whether there is a preference for the accumulation in certain organs and any effect from biopersistence of such ENPs.

Conclusion

It can be summarized from the reported genotoxicity data that the characterization of ENPs is a crucial part in predicting the genotoxic potential of ENPs. It is also suggested to perform an array of cytotoxicity and genotoxicity tests before predicting the adverse effect of ENPs, as there are knowledge gaps in assessing the human risk to ENPs exposure (Fig. 4). Also, the interference/binding of ENPs with the test methods and enzymes should be cross-checked during the experiments. Despite having existing uncertainties about the test methods, the findings on the carcinogenic potential of some ENPs should be taken seriously. It is recommended that the assessment should focus on the extent to which the human can be exposed to nanomaterials on daily basis. Also the multidisciplinary approaches using different models (from in silico to in vivo) and test methods should be used in assessing the ENPs-associated risk (Fig. 5). At the same time, the valid methods on all the possible routes of exposure (inhalation, dermal, oral) to determine the long-term effect of ENPs should be developed. Under the present circumstances, carcinogenic hazards and the potential health risks of ENPs should also be differentiated based on the specific physico-chemical properties and effective concentration of the specific ENPs.

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