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REVIEW

The Biological Mechanisms and Physicochemical Characteristics Responsible for Driving Fullerene Toxicity

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This review provides a comprehensive critical review of the available literature purporting to assess the toxicity of carbon fullerenes. This is required as prior to the widespread utilization and production of fullerenes, it is necessary to consider the implications of exposure for human health. Traditionally, fullerenes are formed from 60 carbon atoms, arranged in a spherical cage-like structure. However, manipulation of surface chemistry and molecular makeup has created a diverse population of fullerenes, which exhibit drastically different behaviors. The cellular processes that underlie observed fullerene toxicity will be discussed and include oxidative, genotoxic, and cytotoxic responses. The antioxidant/cytoprotective properties of fullerenes (and the attributes responsible for driving these phenomena) have been considered and encourage their utilization within the treatment of oxidant-mediated disease. A number of studies have focused on improving the water solubility of fullerenes in order to enable their exploitation within biological systems. Manipulating fullerene water solubility has included the use of surface modifications, solvents, extended stirring, and mechanical processes. However, the ability of these processes to also impact on fullerene toxicity requires assessment, especially when considering the use of solvents, which particularly appear to enhance fullerene toxicity. A number of the discussed investigations were not conducted to reveal if fullerene behavior was due to their nanoparticle dimensions but instead addressed the biocompatibility and toxicity of fullerenes. The hazards to human health, associated with fullerene exposure, are uncertain at this time, and further investigations are required to decipher such effects before an effective risk assessment can be conducted.

Key Words: **fullerene; carbon; nanoparticle; nanotoxicology; tetrahydrofuran.**

The realization that the field of nanotechnology has the ability to provide many benefits to society, as well as financial gains, has prompted the rapid growth of the types and quantities of available nanomaterials (defined as having one dimension less than 100 nm; British Standards Institution [BSI] Report, 2007) that are anticipated to be exploited in numerous diverse applications. A number of reports (see, e.g., Maynard *et al.*, 2006) have called for an improved understanding of the potential detrimental implications for human health that are associated with the rapid development of this field. Therefore, revealing the risks (comprising of exposure and hazard assessments) of such nanomaterials for humans is of paramount importance. In particular, as nanomaterials are a diverse population of materials that vary with regards to their size, shape, and composition, identification of nanomaterial attributes that may be associated with adverse health effects is essential. This will enable the safe development and integration of nanomaterials into products. Such knowledge will also be useful in managing risk in the future by allowing control measures to be introduced for minimizing exposure to hazardous nanomaterials, perhaps through the development of protective measures, or by the use of alternative materials. This would therefore allow safety to be built into the design of nanomaterials and their applications and demonstrates that the field utilizes a responsible research approach. This hazard review, relating to the toxicity of fullerenes, was adapted from a series of reviews conducted as part of the Engineered Nanoparticles: Review of Health and Environmental Safety (ENRHES) project, funded by the European Commission FP7 funding program (http://nmi.jrc.ec.europa.eu/project/ENRHES.htm). The project aimed to conduct a comprehensive and critical review of the available health and environmental safety data. Studies that are commonly employed to determine the human toxicity of a wide variety of nanomaterials include *in vivo* (within mice and rats) and *in vitro* (using cell lines and primary cells) models. Such investigations relating to fullerene toxicity will be discussed within this review. The information used in this review

is to be combined with a review of the human and environmental published exposure data and an evaluation of industrial activity in this area in order to provide the basis for a risk assessment based on current understanding.

Carbon fullerenes (also termed C_{60}, Buckminsterfullerene, or buckyballs) were first discovered by Kroto *et al*. (1985) and describe 60 linked carbon atoms in a highly stable icosahedron, consisting of 60 vertices and 32 (12 pentagonal and 20 hexagonal) faces (see Nielson *et al*., 2008 for more details). Consequently, carbon fullerenes have a spherical cage-like structure and have a diameter of about 1 nm and thus can be defined as a nanomaterial. Fullerene production can occur naturally as they can be released from combustion processes such as forest fires (Powell and Kanarek, 2006). Alternatively, there has been an increase in the intentional production of fullerenes due to the realization that novel exploitable properties are exhibited by materials that contain "nano" dimensions (< 100 nm), and as a result, the use of C_{60} is being considered for drug delivery and recently within a number of cosmetic products, such as face creams (Halford, 2006). Fullerenes therefore conform to the definition of a nanomaterial, whereby they contain a dimension of less than 100 nm (BSI Report, 2007), which gives rise to unique novel properties to enable their exploitation within numerous applications. Therefore, assessing the toxicity of fullerenes is necessary as, although beneficial properties are exhibited by nanomaterials as a consequence of their small size, unpredictable toxic effects may also transpire that require identification. However, it is relevant that a number of the described studies are relatively old, and so their focus was not on the nano dimensions of fullerenes, and instead were preliminary investigations into fullerene toxicity and biocompatibility. A particular focus of the more recent studies has been to determine the antioxidant properties exhibited by fullerenes due to the fact that this phenomenon can be exploited within the treatment of oxidant-mediated disease. Studies have also investigated methods to improve fullerene dispersion within aqueous suspensions to enable their utilization within biological systems.

Although C_{60} is depicted as particles consisting of a cage of 60 or more carbon atoms, in reality, fullerene molecules crystallize into larger structures. Therefore, exposure is often to clusters of crystals termed nano or colloidal fullerenes. In order to enhance dispersion, and to minimize the cluster/crystal size, it is common to use chemical or physical means of particle dispersion. Due to the importance of fullerene solubility to its exploitation, fullerene derivatives have been generated that exhibit greater water solubility than their "pristine" (i.e., unmodified) counterparts. For example, fullerols (also termed fullerenols) are often produced, whereby the surface of the fullerene molecule is polyhydroxylated to render fullerenes more water soluble. Therefore, numerous fullerene types exist, and their impact on fullerene properties and toxicity requires identification. However, despite the use of chemical or physical procedures to aid in particle dispersal, the stability of such suspensions is uncertain and is likely to impact on the toxicity exerted by fullerenes.

Ascertaining which attributes of fullerenes drive the observed toxic responses, as well as identifying the mechanisms underlying any observed toxicity, is of vital importance. Experimental exposure conditions are described within the review and include preparation techniques utilized, dispersion methods employed, model used, concentration of particles administered, and duration of exposure. However, such information is not always clearly stipulated by investigators, which is a disadvantage when assessing the contribution of experimental design to the observed toxicity of fullerenes. In addition, identifying hazards related to fullerene exposure is complicated by the fact that there are a variety of fullerene derivatives available, which stems from the number of carbon atoms used to generate fullerenes, the diverse array of moieties that can be attached to the fullerene surface, and the different preparation processes utilized to render fullerenes water soluble. Therefore, thorough physicochemical characterization of fullerenes is required to accompany the toxicological observations made (Warheit, 2008) in order to assess what attributes of fullerenes are responsible for driving the observed toxic responses. Specifically, determining particle characteristics that are repeatedly associated with toxic responses would facilitate the design of nanomaterials, which avoid the incorporation of such attributes and thus allow their safer integration into products to allow the benefits of nanotechnology to be realized.

IN VIVO ASSESSMENT OF FULLERENE TOXICITY

As a consequence of the number of expected applications that contain fullerenes, it is anticipated that exposure could occur via oral, dermal, pulmonary, or injection routes. Therefore, toxicity at the site of exposure is of particular interest (namely the skin, lungs, and gastrointestinal tract), but it is also relevant that fullerenes may distribute throughout the body and accumulate within sites distal to their portal of entry, such as the liver and spleen. The *in vivo* studies identified have either administered fullerenes via the lungs, via injection (iv or ip administration), or dermally. These studies are few in number, and so the available data relating to *in vivo* toxicity are rather limited. It is necessary to highlight that a particular focus on pulmonary and dermal exposures to fullerenes has been used in this review, with injection studies used to address the toxicokinetics and biocompatibility of fullerenes.

Pulmonary Exposure to Fullerenes

Previous studies have highlighted that particle size is an important determinant of particle toxicity, specifically that particles with nano dimensions (< 100 nm) are more toxic than their larger equivalents (see, e.g., Ferin *et al*., 1992), and the relevance of this to fullerenes is worthy of consideration due to their definition as nanoparticles (NPs). Accordingly, Baker *et al*. (2008) exposed rats to NP (55 nm diameter, 2.22 mg/m^3) and

microparticle (0.93 μm diameter, 2.35 mg/m^3) forms of C_{60} via nasal inhalation. Briefly, aerosol generation involved the milling of bulk fullerenes, which were transferred into a nitrogen gas stream, then a particle attrition chamber was used to reduce particle size, NPs only were then heated and flash vaporized and oxygen levels were increased prior to their exposure of animals. The exposures were conducted for 3 h/day for 10 consecutive days, and toxicological assessments were conducted up to 7-day postexposure. The lung burden of particles was also assessed, which was generally greater for the NP-exposure group. Specifically, the pulmonary deposition fraction of C_{60} NPs was 14.1% and for C_{60} microparticles was 9.3%. However, the half-life for C_{60} within both treatment groups was similar, being 26 days for NPs and 29 days for microparticles, thus suggesting that similar elimination processes were involved during their removal from the lungs. The exposures did not result in detectable gross or microscopic lesions at necropsy, and minimal hematology and serum chemistry changes were observed. Within the lungs, no cellular infiltration (indicative of an inflammatory response) was observed, although C_{60} was internalized by alveolar macrophages. Therefore, the study did not reveal any inflammation or toxicity for C_{60} in the lungs of rats nor did it reveal any differences in toxicity when generated in the NP or microparticle forms.

Fujita *et al.* (2009) treated rats with C_{60} via whole-body inhalation for 6 h/day, 5 days/week, for a total of 4 weeks. The exposure system consisted of a pressurized nebulizer and a mist dryer, connected to the exposure chamber. Observations continued for a period of up to 1-month postexposure, during which time the authors observed, using DNA microarrays, an upregulation in a small number of genes involved with the stimulation of inflammation, oxidative stress, apoptosis, and metalloendopeptidase activity. C_{60} was also observed within alveolar macrophages and epithelial cells. However, the authors concluded that the inflammatory response and tissue injury induced were not severe in magnitude, despite the fact that only gene changes were measured. Similarly, Sayes *et al.* (2007) reported that no pulmonary toxicity was associated with intratracheal exposure to C_{60} or $C_{60}(OH)_{24}$ of rats (up to 3 mg/kg for a period of up to 3 months following exposure), which is in contrast to the response induced by α-quartz that was proinflammatory and profibrotic.

Many studies have demonstrated that a range of NPs induce proinflammatory effects in the lung (see, e.g., Donaldson and Stone, 2003 for a review), but a study by Roursgaard *et al.* (2008) assessed the anti-inflammatory potential of fullerols at doses of 0.02–200 μg per mouse (equivalent to 0.001–10 mg/kg in saline). This was achieved by evaluating their ability to attenuate the pulmonary inflammatory response elicited by α-quartz in mice. In fact, intratracheal exposure to fullerols at a dose of 200 μg (equivalent to 10 mg/kg) elicited a neutrophil-driven pulmonary inflammatory response, which was associated with increased macrophage inflammatory protein-2 production. This inflammatory response was however less pronounced than that for quartz. Mice pretreated with fullerol (< 20 μg [equivalent to 1 mg/kg]) demonstrated an attenuation of the subsequent inflammatory response elicited by quartz. This was proposed by the authors to be due to the ability of fullerols to reduce reactive oxygen species (ROS)-mediated inflammation, but this finding was only relevant with the lower doses of fullerols studied. Therefore, the results implied that at low concentrations, fullerols may have protective anti-inflammatory properties but at higher concentrations they exhibit a proinflammatory response.

Therefore, the findings from these small number of available studies demonstrate that following exposure via the pulmonary route, fullerenes are capable of eliciting localized responses that are generally not inflammatory or toxic but in contrast are anti-inflammatory in nature, with the type of response initiated likely to be reliant on the fullerene in question, exposure method, and the dose used. Consequently, insufficient evidence is currently available to make definitive conclusions about what drives the pro- and anti-inflammatory responses, associated with fullerene pulmonary exposure. No studies were identified that addressed uptake of fullerenes from the lungs to the cardiovascular system.

Intraperitoneal Exposure

Chen *et al.* (1998a) generated fullerene-protein conjugates, using bovine thyroglobulin, bovine or rabbit serum albumin, or derivatives of lysine, and investigated their antigenicity. Mice exposed to the particles ip (in Freund's adjuvant) generated antibodies against the C_{60} derivatives, suggesting that they exhibited antigenic behavior. The findings were expanded upon by Erlanger *et al.* (2001) who demonstrated that anti-C_{60} antibodies were able to interact with single walled carbon nanotubes, which was imaged using atomic force microscopy. The findings *insinuated* that C_{60} derivatives may act as sensitizing agents and thus have the potential to modulate subsequent immune responses.

Chen *et al.* (1998b) administered rats with water-soluble polyalkylsulfonated C_{60} via ip injection (in water) in an acute (up to 1000 mg/kg for 24 h) or a subacute (up to 60 mg/kg, with daily exposures for 12 consecutive days) setting. Specifically, within 24 h, five of six rats died when administered a dose of 750 mg/kg of fullerene, and 100% of exposed rats died in the 1000 mg/kg treatment group. The fullerene was found to have an LD_{50} of 600 mg/kg. The kidney was recognized as a primary site of fullerene elimination and toxicity within the acute study, which was reproduced within the subacute study. In addition, macrophages within the liver and spleen were observed to be laden with particles in the subacute group. Within preliminary studies, liver cytochrome P450 activity was also observed to be suppressed. It is necessary to highlight that exceptionally high doses were utilized within this study (in order to attain an LD_{50} value) that could explain the pathology and mortality that transpired.

In the very limited number of studies that have been conducted, the ip injection of fullerenes has been used to assess fullerene biocompatibility and tissue distribution. It would

appear that fullerenes are able to elicit an antigenic response due to its potential to modulate inflammatory responses, but the applicability of this to other fullerene derivatives requires assessment. In addition, the kidney, liver, and spleen have been demonstrated to be a target of fullerene toxicity, and so their transport within the blood is anticipated following ip injection, but this requires further investigation to determine how universal this finding is to all fullerenes.

Dermal Exposure

Only one investigation studying the potential dermal effects of fullerenes was found. Specifically, Huczko *et al.* (1999) used patch testing to assess the skin irritant potential of fullerene soot within 30 volunteers (who reported irritation and allergic susceptibilities) for a 96-h exposure time. No skin irritation was found.

Studies that purport to study the consequences of dermal exposure to fullerenes are lacking, with the only available investigation suggesting that no detrimental outcome on the skin is apparent, but this requires more extensive investigation, especially due to the exploitation of fullerenes within cosmetic products.

Oral Administration

Yamago *et al.* (1995) investigated the distribution of ^{14}C-labeled water-soluble C_{60} (in saline containing 0.2% Tween 80) within rats, following oral administration, for a period of up to 160-h postexposure. Subsequent to oral exposure, C_{60} was not effectively absorbed, but instead, the majority was excreted in the feces within 48 h. However, it is of interest that trace amounts of fullerene were observed within urine, therefore implying that some fullerenes were able to pass through the gut wall.

Mori *et al.* (2006) used fullerite, a mixture of C_{60} and C_{70} (in 0.5% sodium carboxymethyl cellulose aqueous solution including 0.1% Tween 80), to evaluate the acute toxicity (up to 14 days) of fullerenes, subsequent to the oral exposure of rats, at a dose of 2000 mg/kg. No lethality or other signs of toxicity in terms of behavior or body weight were evident during the observation period, despite the high dose that was administered, with fullerene elimination within feces evident.

Chen *et al.* (1998b) demonstrated that polyalkylsulfonated (water-soluble) C_{60} (in water) was not lethal, subsequent to the oral exposure of rats in acute (50 mg/kg, single administration) or subacute (50 mg/kg daily for 12 days) exposure setups, and as a consequence was considered to be nontoxic. These findings are in contrast with the lethality associated with ip exposure, as mentioned previously. However, perhaps sublethal toxicity should be a focus of future studies.

The limited number of investigations that evaluated the consequences of oral administration suggested that fullerenes are primarily eliminated within feces. However, it has also been suggested that a small but unspecified proportion of the fullerene dose is able to pass through the gut wall and thereby enter the circulation. Such studies are inadequate in number to make definitive conclusions regarding the transfer of fullerenes into the circulation and therefore their systemic availability following oral exposure.

absorption, distribution metabolism excretion (ADME)

Profile of Fullerenes

Determining the kinetics of fullerenes within the body, subsequent to exposure (via the lungs, gut, and skin), is necessary to identify potential targets of fullerene toxicity and thereby direct relevant *in vitro* assessments of their toxicity at particular target sites. This is necessary as the delivery of fullerenes to target organs, such as the liver or kidneys, requires their transfer into blood from their exposure site, and so their likelihood of accessing different sites within the body is of relevance. Accordingly, a number of barriers (at the exposure site and those apparent within secondary targets) are in place to prevent against uptake, and it is necessary to determine if this is surmounted by fullerenes to determine their systemic uptake and therefore availability.

Studies that provide evidence for the absorption of fullerenes into the blood from their exposure site are few in number, and as such, this question should be a focus of future investigations. Baker *et al.* (2008) did not detect fullerenes within the blood, following inhalation by rats, suggesting that they do not translocate from their exposure site. However, this was suggested to occur due to their potential biotransformation within the lung and insensitivity of the detection method. Particles were presumably eliminated due to the action of alveolar macrophages and mucociliary escalator, but this requires further consideration. Perhaps radioisotope or fluorescent labeling could allow for the better detection of fullerenes when evaluating fullerene kinetics. In contrast, Yamago *et al.* (1995) suggested that fullerenes were able to pass into the blood from the gut. Chen *et al.* (1998b) also illustrated that the kidney, liver, and spleen were associated with the toxicity or accumulation of fullerenes following ip injection, which is suggestive of their transport within blood. Targeting of these organs is likely to be driven by the resident macrophage populations that sequester foreign particles.

The metabolism of fullerenes has been suggested to occur following their accumulation within the liver (Gharbi *et al.*, 2005). As yet, the metabolites formed are unspecified, so that their identification requires investigation in the future.

The elimination of fullerenes within urine (Yamago *et al.*, 1995) and feces (Mori *et al.*, 2006; Yamago *et al.*, 1995) has been demonstrated, suggesting that they may be eliminated, in part, from the body following exposure via a number of routes.

Information regarding the absorption, distribution metabolism excretion (ADME) profile of fullerenes is generally lacking and therefore warrants further investigation in future studies. In the small number of studies described here, it would appear that the majority of fullerenes remain at the deposition site (specifically within the lungs and gut) but that it is also possible for fullerenes to cross cell barriers and to be transported within

the blood. Accumulation appears to be predominant within the liver, kidneys, and spleen, with evidence of toxicity also manifesting at these sites of accumulation. Metabolism of fullerenes has also been suggested, and the consequences of this require consideration. Elimination of fullerenes within the feces and urine has also been demonstrated, which may reduce their propensity for distribution and toxicity. However, it is relevant to note that the representative nature of the limited number of findings for all fullerene derivatives is unknown at this time.

Distribution of C_{60} Following Injection

Fullerene distribution following injection into blood has been studied both due to their potential use as carriers for drugs and to assess their distribution and localization sites should they enter the blood via other routes (e.g., following inhalation).

Yamago *et al.* (1995) investigated the distribution of ^{14}C-labeled water-soluble C_{60} within rats after iv injection. Subsequent to exposure, the fullerenes were rapidly removed from the blood (only 1.6% of the administered dose remained in the blood after an hour) and accumulated within the liver, which was the primary site of localization, although some localization was also evident within, e.g., the kidney, lungs, spleen, heart, and brain. In a similar study, Bullard-Dillard *et al.* (1996) also exposed rats via iv exposure to radiolabeled C_{60} (0.2μM, equivalent to 0.144 μg/ml, in PBS). Clearance of C_{60} from the blood was again rapid, with only 1% of the administered dose of pristine C_{60} remaining within the circulation after 1 min. However, the clearance of quaternary ammonium salt–derivatized C_{60} was slower, with 9% of the dose remaining at 1-min postexposure, which was attributed to its more hydrophilic water-soluble character. Again, the majority of the unmodified particles were contained within the liver (more than 90%) at 120-min postexposure, with minimal accumulation within the spleen, lung, and muscle. The water-soluble C_{60} had a wider tissue distribution, with only 50% of the administered dose evident within the liver and the remaining dose contained in the spleen, lungs, muscle, and cellular component of blood. After 120 h, it was apparent that the majority (95%) of unmodified C_{60} still remained within the liver, with no evidence of elimination within urine or feces, highlighting that the liver is a potential target for fullerene accumulation and toxicity.

In line with these findings, Gharbi *et al.* (2005) demonstrated that C_{60} (dispersed using mechanical milling in an aqueous media) was able to accumulate within the liver following the ip exposure of rats, which was also indicated by a color change (to dark brown) of the liver. However, C_{60} localization within the liver decreased with time (nearly all were eliminated by day 13), and so it was suggested that the liver was capable of either eliminating C_{60} (within the feces) or biochemically transforming C_{60} as C_{60} metabolites were identified within the liver. Histological analysis revealed that no inflammation or fibrosis was associated with the hepatic accumulation of particles, which was primarily accounted for by their uptake by Kupffer cells.

The findings from the different studies therefore share the commonality that subsequent to injection, fullerenes preferentially accumulate within the liver. Therefore, it is of high relevance to evaluate the impact of fullerene accumulation on liver function and to assess the contribution of the liver to the metabolism of fullerenes, in addition to considering the ability of the liver to facilitate the removal of fullerenes from the body within bile and therefore the feces.

IN VITRO INVESTIGATIONS OF C_{60} TOXICITY

As for the *in vivo* assessment of fullerene toxicity, there are a limited number of investigations that describe the toxic potential of fullerenes *in vitro*, which have concentrated on the dermal and cardiovascular toxicity of fullerenes.

Dermal Models

Scrivens *et al.* (1994) demonstrated that ^{14}C-labeled C_{60} (1.3μM, equivalent to 0.936 μg/ml, with particles prepared using tetrahydrofuran [THF] and dispersed in serum-free cell culture medium) was internalized by immortalized human keratinocytes so that after a 6-h exposure time, 50% of the applied concentration was contained within the cells. Despite the internalization, C_{60} exposure (20nM–2μM) did not impact on cell proliferation. A similar effect was observed by Bullard-Dillard *et al.* (1996) who observed that C_{60} and quaternary ammonium salt–derivatized C_{60} (up to 2μM, equivalent to 1.44 μg/ml) were internalized by keratinocytes, with the process being slower for derivatized particles. However, it was apparent that C_{60} elicited a decrease in cell proliferation that was evident at high concentrations (2μM) and over an extended period of time of 8 days. Rouse *et al.* (2006) found that phenylalanine-derivatized C_{60} (up to 0.4 mg/ml, dispersed in serum-free culture medium, with vortexing and sonication used to break up aggregates) was internalized by HEK keratinocytes and elicited an inflammatory response, indicated by an increase in interleukin (IL)-6, IL-8, and IL-1β production. The fullerene ultimately initiated dose-dependent cytotoxicity via a necrotic mechanism. These results were expanded upon by Rouse *et al.* (2007) who illustrated that there was a relationship between C_{60} penetration and skin flexing within an *ex vivo* pig skin preparation. Specifically, a fullerene-peptide conjugate (dispersed in PBS) was internalized into epidermal and dermal layers (not reaching microvasculature or blood), and this effect was more pronounced within flexed skin (experienced, e.g., when walking barefoot) than unflexed skin. It is also of interest that the penetration of the particles did not occur via their direct transport through cells but indirectly between skin cells via intercellular spaces. Sayes *et al.* (2004) found that the cytotoxic potential (mediated by lipid peroxidation) of different forms of derivatized fullerenes to human dermal fibroblasts, HepG2 hepatocytes, and normal human astrocytes was dependent on the type and level of functionalization (see below).

The findings from the discussed studies suggest that the fullerene type, skin condition, and experimental protocol (cell type, concentration, and duration) are able to influence the inflammogenic and cytotoxic potential of fullerenes to the skin *in vitro*. No clear conclusion regarding uptake potential or toxicity can be generated for skin at this time, and it is possible that different fullerenes will behave differently in this target organ.

Models of Cardiovascular Effects

As fullerenes may have the potential to translocate from their site of exposure into the circulation, or be directly administered into the blood through injection, they are likely to encounter the endothelial cells that line blood vessels to potentially cause vascular injury. As a result, Yamawaki and Iwai (2006) investigated the ability of $C_{60}(OH)_{24}$ (1–100 μg/ml, dispersed in serum-containing cell culture medium) to induce endothelial damage within the HUVEC cell line. Following an acute exposure (24 h), fullerenes were internalized by cells and elicited a dose-dependent decrease in cell viability, which was suggested to be autophagic (and was demonstrated to be nonapoptotic). Subsequent to a chronic exposure (10 days), fullerenes detrimentally affected cell attachment and slowed cell growth. It was therefore speculated (by the authors) that exposure to fullerenes is a potential risk for cardiovascular disease initiation or progression. However, further investigations *in vivo* would be required to confirm such a suggestion.

Radomski *et al.* (2005) demonstrated that a number of engineered particles and urban particulate matter (0.2–300 μg/ml, dispersed in Tyrode's solution, with sonication used to minimize aggregation) were able to stimulate the aggregation of platelets (to varying extents) after an 8-min exposure. However, C_{60} was not effective in this assay, suggesting that they are relatively less thrombogenic than other NPs.

The limited number of available investigations provided conflicting results regarding the prothrombogenic potential of fullerenes.

Additional Targets

Interest in investigating the ocular toxicity of fullerenes derives from the potential to exploit fullerenes as drug carriers that bypass blood-ocular barriers to enable their delivery to the blood (Roberts *et al.*, 2008). Fullerols ($C_{60}(OH)_{22–26}$) have been observed to accumulate within human HLE-B3 lens epithelial cells in *in vitro* and *ex vivo* models, and this accumulation was associated with cytotoxicity (Roberts *et al.*, 2008). The cytotoxicity of fullerols was observed to be enhanced with ultraviolet A (UVA) and visible light exposure during treatment, illustrating that there is a photosensitive aspect to fullerol toxicity. The endogenous antioxidant lutein was able to offer some protection against the photo-oxidative cytotoxicity induced by fullerol, thus suggesting an ROS component to the response. However, this was not conclusive since neither ascorbic acid nor N-acetylcysteine (NAC) antioxidants could achieve the same effect. It was also observed that fullerol was able to bind to the lens protein α-crystalline (which is likely to increase its retention within cells) so that interactions with biological molecules is a realistic possibility. Consequently, the potential for fullerene internalization, enhanced ROS production, and interactions with cellular components were highlighted within this study.

However, it is of interest that Huczko *et al.* (1999) used the Draize rabbit eye irritation test to reveal the potential toxicity of fullerenes to the eye. Instillation of a fullerene soot suspension (for up to 72 h) was observed to have no toxicity within the eye.

THE BIOLOGICAL MECHANISMS DRIVING FULLERENE TOXICITY

A number of investigators have demonstrated that fullerenes are capable of eliciting toxicity that is mediated via the stimulation of an inflammatory response and the involvement of oxidative stress. Therefore, it may be possible to generate broad conclusions regarding the mechanisms underlying fullerene toxicity. Processes underlying fullerene toxicity will therefore be discussed. In addition, the uptake of fullerenes by cells will be addressed as this has the ability to promote not only their clearance but also their toxicity. The genotoxic and reproductive toxicology of fullerenes will also be considered.

Fullerene-Mediated Inflammatory Responses

NPs, of a variety of types, have been demonstrated to induce inflammation, and so it is often believed to be a common response associated with exposure (for reviews, see, e.g., Donaldson and Stone, 2003; Donaldson *et al*, 2005; Kagan *et al*., 2005). As such, the proinflammatory potential of fullerenes requires consideration due to their definition as NPs. There are *in vitro* investigations that indicate that an inflammatory response may be instrumental to the toxicity of fullerenes, as demonstrated by the enhanced production of proinflammatory mediators, such as IL-8 and tumor necrosis factor α (TNFα) (see, e.g., Rouse *et al*., 2006). However, there is a lack of information available regarding *in vivo* inflammatory-mediated responses, which should be a focus of future experiments. Furthermore, a concentration-dependent effect is likely as Roursgaard *et al*. (2008) demonstrated that fullerol has an anti-inflammatory effect within the mouse lung at lower doses but a proinflammatory effect at higher concentrations within mice.

Some studies suggest that fullerene derivatives may in fact be capable of suppressing inflammatory responses. Huang *et al*. (2008) generated C_{60}-based fulleropyrrolidine-xanthine molecules (dispersed in cell culture medium containing 1% dimethyl sulfoxide [DMSO]). It was anticipated that the fullerene component would act as a free radical scavenger and the xanthine attachment would be capable of suppressing inflammatory reactions. Pretreatment of lipopolysaccharide (LPS)-stimulated J774 macrophage–like cells with the fullerene was effective at scavenging LPS-induced nitric oxide and TNFα production. These findings therefore suggest that

fullerene derivatives could be exploited as anti-inflammatory agents. However, more work is required to investigate this hypothesis further.

Additionally, Tsao *et al.* (1999) demonstrated that carboxyfullerene (2 mg/ml) pre- or posttreatment (up to 40 mg/kg in PBS) was able to attenuate *Escherichia coli*–mediated meningitis within mice following ip injection. It was suggested that *E. coli*-induced inflammation increased permeability of the blood-brain barrier, thus permitting the access of fullerenes into the brain in order to enable their protective behavior to emerge. Carboxyfullerenes may therefore have a protective effect against bacterial meningitis, which was more effective than dexamethasone (anti-inflammatory steroid) treatment. However, it is worth highlighting that the concentrations of fullerenes used within this experiment were high.

Harhaji *et al.* (2008) investigated the impact of fullerene treatment on TNFα-mediated cell death. It was observed that C_{60}/C_{70} (prepared using THF and dispersed in serum-containing cell culture medium) and polyhydroxylated fullerene preparations (up to 250 μg/ml for 24 h, dispersed in serum-containing cell culture medium) were cytotoxic to the mouse L929 fibroblast cell line but that C_{60}/C_{70} was more potent. Furthermore, a combined treatment of C_{60}/C_{70} with TNFα was more toxic than observed for each treatment alone, thus suggesting a synergistic interaction. Paradoxically, it was evident that a cotreatment of polyhydroxylated fullerene with TNFα was able to reduce the cytotoxic effect of TNFα, thus *insinuating* that functionalized fullerenes acquired a protective activity. This finding was further supported by the observation that C_{60}/C_{70} exposure enhanced, and polyhydroxylated fullerenes prevented, TNFα-mediated ROS production and mitochondrial depolarization. It was speculated that the capacity of fullerenes to modulate TNFα-mediated toxicity was dictated by their ability to modulate TNFα-mediated ROS production. Specifically, C_{60}/C_{70} was suggested to enhance ROS production, increasing the cytotoxic response associated with TNFα exposure, whereas polyhydroxylated preparations attenuated ROS production, and thereby had a cytoprotective effect, by antagonizing TNFα-mediated cytotoxicity. The study therefore highlighted that two fullerene preparations can behave very differently, which is a logical conclusion but means that it is difficult to make generalizations about fullerene behavior and therefore predict their behavior. However, it is relevant that the inclusion of THF within the preparation of the C_{60}/C_{70} sample may contribute to the greater toxicity of this sample (see below).

It is often assumed that NPs stimulate a response that is inflammatory, and this has been demonstrated, to a very limited extent, for fullerenes. However, in contrast, the anti-inflammatory behavior exhibited by fullerenes has been a focus of investigations due to opportunity to exploit this phenomenon within therapeutic interventions. The findings indicate that the concentration of fullerene, the fullerene derivative in question (which, on occasions, were purposefully altered to integrate an anti-inflammatory aspect), and experimental model used are able to impact on the inflammatory potential of fullerenes.

Fullerene-Mediated Oxidative Responses

The ability of NPs to enhance ROS production within cells, and thereby stimulate the development of oxidative stress (see, e.g., Stone *et al.*, 1998), has prompted investigations to determine whether fullerenes have the same pro-oxidant potential due to their classification as NPs.

Sayes *et al.* (2005) demonstrated that *nano*C_{60} (0.24–2400 ppb, equivalent to 0.00024–2.4 μg/ml, prepared using THF and then dispersed in serum-containing cell culture medium) exerted cytotoxicity that was mediated through enhanced ROS production, lipid peroxidation, and membrane damage in a variety of cell lines (dermal fibroblasts, hepatocytes, and astrocytes). The damage to cell membrane integrity was confirmed by evidence that lactate dehydrogenase (LDH) was released from cells and that fullerene-exposed cells were more permeable to dextran. The involvement of ROS was confirmed by the observation that the administration of the antioxidant ascorbic acid could prevent against the appearance of fullerene-mediated cytotoxicity. Similar observations were made by Kamat *et al.* (2000) who observed that C_{60} and $C_{60}(OH)_{18}$ could elicit membrane damage under photosensitive conditions, which was accounted for by the appearance of lipid peroxidation within isolated rat liver microsomes. Furthermore, the oxidation of proteins indicated by the formation of protein carbonyls, depletion of membrane enzymes, and attenuation of the toxic response using antioxidants all provided further confirmation of an oxidant-driven response. Although both fullerene species were capable of eliciting a pro-oxidant response, the toxicity was greater for $C_{60}(OH)_{18}$ than for C_{60}.

Paradoxically, Xia *et al.* (2006) illustrated that fullerol ($C_{60}(OH)_{22–26}$, dispersed in serum-containing cell culture medium) exposure was incapable of stimulating ROS production, depletion of glutathione (GSH), or stimulation of heme oxygenase 1 expression within RAW 264.7 macrophages, despite the fact that it was a powerful ROS producer in cell-free conditions and that the fullerols were internalized by cells. No TNFα production was associated with fullerol exposure, but an increase in mitochondrial calcium levels was observed, which implied that mitochondrial damage occurred, although no changes in mitochondrial membrane potential were realized. A panel of ambient and manufactured NPs were tested within the same study, and importantly, it was recognized that they differed in their ability to be internalized, stimulate ROS production, deplete cellular antioxidants, and induce mitochondrial toxicity and cytotoxicity.

The findings outlined are often contradictory (summarized in Table 1) and suggest that in some conditions, fullerenes may induce pro-oxidant effects, but in others they do not, and this is likely to be dictated by the fullerene in question, the cell type being investigated, and the experimental setup.

TABLE 1
The Pro-oxidant Activity of Fullerenes

Paper	Fullerene species	Model	End points assessed	Observation	Conclusion
Kamat *et al.* (2000)	C_{60} and $C_{60}(OH)_{18}$ (and photo-oxidation)	Rat liver microsomes	Lipid peroxidation products (conjugated dienes, lipid hydroperoxides, thiobarbituric acid–reactive substances)	Lipid and protein oxidation evident	Phototoxicity of fullerene derives from oxidative damage
			Protein damage: oxidation (protein carbonyls) and inactivation of enzymes	Antioxidants protected against fullerene-induced oxidative damage	Damage to lipid and proteins greater for $C_{60}(OH)_{18}$
			Protective effect of various free radical scavengers investigated		
Sayes *et al.* (2004)	*Nano*C_{60}, $C_{60}(OH)_{24}$ C_3, Na+2-3[C_{60}O7-9(OH)12-15](2-3)-	HDF and HepG2 cell lines	LDH and live/dead assays assessed cell death	*Nano*C_{60} induced greatest superanion production in acellular conditions	↑ Water solubility = ↓ cytotoxicity
			Acellular determination of superanion production	*Nano*C_{60} had greatest cytotoxic potential	Cytotoxicity of *nano*C_{60} due to oxidative damage to the cell membrane
			Xathine oxidase activity used to assess ROS production		
Sayes *et al.* (2005)	*Nano*C_{60}	HDF, NHA, and HepG2 cell lines	LDH and live/dead assays assessed cell death	*Nano*C_{60} induces cell death	*Nano*C_{60} toxicity is driven by an oxidant-mediated response
			Cellular GSH	↑ GSH indicating a protective response	
			Lipid peroxidation (MDA)	↑ lipid peroxidation	
			Protective effect of NAC pretreatment determined	NAC protects against *nano*C_{60}-induced cell death	

Note. HDF, human dermal fibroblasts; MDA, malondialdehyde; NHA, normal human astrocyte.

Antioxidant Properties of Fullerenes

There has been a focus on investigating the potential free radical–scavenging activity of C_{60}, which has prompted some fullerene derivatives to be described as "radical" sponges (Xiao *et al.*, 2006). This is driven by the knowledge that fullerene administration may be exploited to protect against radical-mediated damage that is associated with toxicant exposure or a number of disease states. A summary of the antioxidant behavior of fullerenes can be observed in Table 2.

Wang *et al.* (1999) demonstrated that lipid-soluble and water-soluble C_{60} derivatives prevented superoxide- and hydroxyl radical–initiated lipid peroxidation within rats to a greater extent than the natural antioxidant vitamin E. While Dugan *et al.* (1996) observed that fullerols were potent antioxidants and were able to decrease excitotoxic-mediated neuronal cell death exerted by N-methyl-D-aspartic acid or α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate.

Gharbi *et al.* (2005) observed that the pretreatment of rats with C_{60} (0.5–2 g/kg) via ip injection protected the liver from carbon tetrafluoride–mediated liver damage, with the free radical–scavenging activity of C_{60} assumed to drive the protective effect observed. It was also suggested that the antioxidant potential of C_{60} is dependent on its degree of dispersion. The authors suggested that aggregates of C_{60} will not exhibit antioxidant properties due to the lack of availability of the unsaturated bonds contained within the molecule's structure so that the antioxidant behavior of fullerenes is improved within water-soluble forms. The authors did not discuss the potential for C_{60} to bind to carbon tetrafluoride, therefore reducing its bioavailability. A decrease in aggregate size would also increase particle surface area, which would make this rather nonspecific mechanism even more potent. In addition, the authors highlighted that it was necessary to inject exceptionally high doses of C_{60} in order to obtain a reproducible level of accumulation within the liver and thereby allow the exertion of its protective effects. However, as no toxicity was associated with this exposure, the use of such high doses was justified ethically but not perhaps in terms of relevancy.

In a different study, Yin *et al.* (2009) investigated the free radical–scavenging activity and therefore cytoprotective properties of a number of fullerene derivatives (dispersed in serum-containing cell culture medium). It was observed that a gadolinium-based metallofullerol (fullerene cage encapsulates metal) Gd@$C_{82}(OH)_{22}$, $C_{60}(OH)_{22}$, and $C_{60}(C(COOH)_2)_2$

TABLE 2
The Antioxidant Behavior Exhibited by Fullerenes

Paper	Fullerene species	Model	End points assessed	Observation	Conclusion
Bogdanovic *et al.* (2008)	$C_{60}(OH)_{24}$	X-ray irradiation of K562 erythroleukemia cells	Cell viability (trypan blue) Morphological alterations (light microscopy) Enzyme activity: GTT, SOD, and GPx	$C_{60}(OH)_{24}$ pretreatment Improved cell viability Reversed the upregulation in GTT caused by irradiation ↑ SOD and GPx activity	$C_{60}(OH)_{24}$ pretreatment maintains redox homestasis directly (due to action as free radical scavenger) or by stimulating an increase in antioxidant enzyme activity
Gharbi *et al.* (2005)	C_{60}	CCl_4 (liver) toxicity in rats	Macro- and microscopic analyses of liver Serum ALT activity Antioxidant status	C_{60} pretreatment Improved appearance of CCl_4 liver (reduction in inflammation and necrosis) Improvement in liver function Circulating vitamin C and E levels restored to normal (indicates absence of oxidative stress) ↑ Liver GSH levels	Protection against CCl_4 free radical–mediated liver damage exhibited by C_{60}
Injac *et al.* (2009)	$C_{60}(OH)_{24}$	Doxorubicin-induced cardio- and hepatotoxicity in rats with colorectal cancer	Macro- and microscopic analyses of liver and heart Antioxidant status Electrocardiogram alterations Serum enzyme/biochemical indicators	$C_{60}(OH)_{24}$ improved the macro- and microappearance of the cardiovascular and hepatic injury Protective effect of $C_{60}(OH)_{24}$ on doxorubicin alterations to cardiovascular depolarization and repolarization ↑ GSH/GSSG ratio, lipid peroxidation (MDA), and protein oxidation reduced by $C_{60}(OH)_{24}$	Protective effect of $C_{60}(OH)_{24}$ against doxorubicin toxicity is better than that exhibited by vitamin C
Lin *et al.* (2002)	Carboxyfullerene	Ischemia-reperfusion injury in the rat brain	Infarct area GSH levels Lipid peroxidation (MDA)	Fullerene pretreatment (local administration) ↓ Infarct area Protection against GSH depletion and lipid peroxidation	Local not systemic treatment effective at protecting against infarction
Trajkovic *et al.* (2007)	$C_{60}(OH)_{24}$	Radiation of rats with x-rays	Survival following a lethal dose of x-rays Blood cell counts (leukocytes) Tissue histology (heart, liver, lung, small intestine, and spleen)	$C_{60}(OH)_{24}$ pretreatment ↑ Survival ↑ Leukocyte numbers (that were ↓ with irradiation) Protection against tissue damage	$C_{60}(OH)_{24}$ is an effective radioprotective agent
Tykhomyrov *et al.* (2008)	Hyd C_{60}	Alcohol-induced brain injury in rats	Open field test LPO Protein carbonylation GFAP expression (marker of astrocyte damage)	Fullerene pretreatment Improved locomotion and behavior ↓ alcohol-mediated increase in LPO and carbonylated proteins Protection of astrocytes against alcohol damage (↓ GFAP)	Protective effect of fullerenes against oxidant-mediated damage within the brain

TABLE 2—*Continued*

Paper	Fullerene species	Model	End points assessed	Observation	Conclusion
Yin *et al.* (2009)	$Gd@C_{82}(OH)_{22}$	H_20_2-induced oxidative injury within A549 lung epithelial and rat brain capillary endothelial cells	Electron spin resonance spin trap (cell-free detection of superoxide anion, hydroxyl, and singlet oxygen radicals) Cell viability (MTT assay) Mitchondrial damage (JC-1 dye) Intracellular ROS production (DCFH assay) Lipid peroxidation inhibition within liposomes Impact on H_2O_2-induced cytotoxicity	Fullerenes protect cells against H_20_2-induced cell death, intracellular ROS production, and mitochondrial damage Fullerenes can scavenge free radicals and ↓ LPO	Protective effects of fullerenes dependent on ability to scavenge ROS, which is increased by greater derivatization
	$C_{60}(OH)_{22}$				
	$C_{60}(C(COOH)_2)_2$				
Xiao *et al.* (2006)	PVP-wrapped C_{60}	UVA-mediated oxidative damage to HaCaT keratinocyte	Cell viability (WST-1) Cell morphology (light microscopy)	C_{60} did not induce cytotoxicity C_{60} restored the cell viability of cells exposed to UVA (most effective as a pretreatment but also exhibited protective effects when exposed during the UVA exposure)	Cytoprotective effect of fullerene against UVA due to its ''radical sponge'' behavior

Note. ALT, alanine aminotransferase; DCFH, dichlorofluorescin; GFAP, glial fibrillary acidic protein; GPx, glutathione peroxidase; GSSG, oxidised glutathione; GTT, γ glutamyl transferase; H_20_2, hydrogen peroxide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; LPO, lipid peroxidation; MDA, malondialdehyde; PVP, polyvinylpyrrolidone; SOD, superoxide dismutase; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium.

all aggregated so that the average size of the particles contained within the aqueous suspensions was 78 nm ($Gd@C_{82}(OH)_{22}$), 123 nm ($C_{60}(OH)_{22}$), and 170 nm ($C_{60}(C(COOH)_2)_2$) and that the extent of aggregation dictated their antioxidant behavior. Specifically, the electron spin resonance spin trap technique was used as an *in vitro* cell-free method to show that the fullerene derivatives could efficiently scavenge a number of free radicals. It was then demonstrated that hydrogen peroxide–mediated cytotoxicity to A549 cells and rat brain capillary endothelial cells was reduced by a pretreatment with the derivatized fullerenes (up to 100μM), with $C_{60}(C(COOH)_2)_2$ being the least protective and $Gd@C_{82}(OH)_{22}$ exhibiting the greatest protective effect. Yin *et al.* (2009) concluded that fullerene derivatives exerted antioxidant-like behavior and that suspensions of ($C_{60}(C(COOH)_2)_2$) contained larger sized particle aggregates that limited their antioxidant potential. This observation therefore supports the suggestion that better dispersed C_{60} suspensions exhibit a more effective antioxidant capacity. The therapeutic exploitation of the antioxidant properties exhibited by $Gd@C_{82}(OH)_{22}$ has also been explored *in vivo*, where its administration was been demonstrated to inhibit the growth of malignant tumors within mice, following ip administration, and that this was due to their ROS-scavenging activity (Yin *et al*., 2008).

Xiao *et al*. (2006) prepared a polymer-wrapped (polyvinylpyrrolidone) fullerene and named it a radical sponge. It was found that treatment of the cells with the fullerene derivative (up to 75μM, equivalent to 54 μg/ml, dispersed in serum-containing cell culture medium), prior to UVA irradiation, was able to protect HaCaT keratinocyte cells from UVA-mediated cytotoxicity, thus demonstrating their cytoprotective behavior. Further investigations revealed that this antioxidant effect was a consequence of the ROS-scavenging activity of the fullerene derivative and not due to its UVA-sheltering or UVA-absorbing activity. Similarly, Lin *et al*. (2002) observed that the systemic administration of carboxyfullerenes (6 mg/kg) prior to ischemic-reperfusion injury of the rat brain was unable to impact on the size of the infarction area that developed, perhaps due to its inability to cross the blood-brain barrier. In contrast, local pretreatment with carboxyfullerenes (up to 0.3 mg) was associated with a reduction in infarct area and lipid peroxidation associated with ischemia-perfusion. However, the local administration of 0.3 mg fullerene was associated with toxicity, namely adverse behavioral changes and in a few cases death. Therefore, the neuroprotective antioxidant properties of carboxyfullerenes were demonstrated, but investigation into the toxic response associated with fullerene exposure requires further assessment as this may limit their therapeutic exploitation within the treatment of ischemic-reperfusion injury. Tykhomyrov *et al*. (2008) also investigated the neuroprotective effects exhibited by fullerenes, whereby they coadministered water-soluble hydrated fullerenes to rats (30nM, equivalent to 21.6 ng/ml, in drinking water) along with alcohol. The fullerenes protected against the oxidative damage within brain tissue that is associated with chronic alcohol exposure. In addition, Mori *et al*. (2007) demonstrated that fullerenes could be used as an effective pretreatment in reducing the lethality

associated with methamphetamine and morphine co-exposure of mice, which is also known to occur via oxidative stress. This protective effect was equivalent or superior to that of more traditional treatments, including cooling and the administration of phospholipase 2 inhibitors.

The use of fullerenes as therapeutic agents has also been explored in relation to their antioxidant-driven cytoprotective behavior. Injac *et al*. (2009) investigated the protective effect of fullerol ($C_{60}(OH)_{24}$) pretreatment (25, 50, and 100 mg/kg in saline with DMSO following ip injection) on doxorubicin toxicity (which is oxidant mediated) within the heart and liver of rats with colorectal cancer. The protective effect of fullerol was witnessed within macro- and microscopic observations, electrocardiogram evaluation, serum biomarkers for myocardial or hepatic damage, and oxidative stress development. In general, an improvement in doxorubicin-associated toxicity was observed within the heart and liver with fullerol pre-exposure. However, lower doses of fullerols exhibited a greater protective effect, and this may be accounted for by the fact that higher doses of fullerols were less well absorbed from the gut or that the high doses administered contributed to the toxicity that was apparent. Therefore, it was again demonstrated that fullerols are able to exhibit protective effects against oxidative-mediated injury, thereby promoting their exploitation as antioxidants, and that this was a dose-dependent phenomenon. Trajkovic *et al*. (2007) investigated the protective effects of fullerol (10 and 100 mg/kg, dispersed in distilled water, for a period of up to 30 days), administered via ip injection, against ionizing radiation within rats and compared this to the traditional radioprotector amifostine. Fullerol pretreatment was able to improve survival rates within rats. The radioprotective effect exerted by fullerols was most pronounced at a dose of 100 mg/kg and was comparable to that of amifostine. As the harmful effects of radiotherapy are known to be mediated by ROS, fullerols were anticipated to be effective radioprotectors by a mechanism that was antioxidant driven. The potential therapeutic exploitation of fullerols was addressed further by Bogdanovic *et al*. (2008) who assessed the ability of $C_{60}(OH)_{24}$ to protect against ionizing radiation–mediated ROS production *in vitro*. The protective effects of fullerol (10μM, equivalent to 11.28 μg/ml, dispersed in serum-containing cell culture medium) were investigated within irradiated, malignant cultured K562 erythroleukemia cells. The survival rate of irradiated cells was improved by fullerol pretreatment, which was suggested to occur due to increased antioxidant defenses within irradiated cells that counteracted the oxidative damage associated with radiation that acted to preserve cell viability.

Although the free scavenging activity of fullerenes is accepted as being potentially beneficial, in certain circumstances, it can be problematic. For example, Ueng *et al*. (1997) observed that a single ip injection of fullerol, at concentrations of 0.1, 0.5, and 1 g/kg (in water), induced mortality of 10, 22, and 54%, respectively, within 3 days of the treatment. Fullerol administration, of greater than 0.5 g/kg, also elicited a decrease in cytochrome P450 content and activity within liver microsomes (isolated from fullerol-exposed animals). The mechanism of this decrease is not known but could be due to the electron-scavenging behavior of polyhydroxylated C_{60} or binding of fullerol to the enzyme thus promoting enzyme destruction or prevention of enzyme synthesis as a result of cell injury. In addition, mitochondrial function was observed to be diminished by fullerol exposure, as indicated by the suppression of oxidative phosphorylation, which is likely to derive from a reduction in the transfer of electrons. However, the high doses used are likely to account for the mortality and cell injury observed and are unlikely to be encountered by humans.

Overall, the studies relating to antioxidant properties of fullerenes suggest that contrary from being toxic, C_{60} and its derivatives could actually exhibit beneficial health effects. However, it appears that the antioxidant properties exhibited by fullerenes are restricted to particular fullerene forms and therefore a number of conditions being met in order to allow its manifestation. Water solubility is likely to impact on their antioxidant/cytoprotective potential so that the better dispersed the fullerene is, the more likely it is that it will exert free radical–scavenging activity, which is likely to derive from their derivatization as they are specifically generated to improve fullerene water solubility. The concentration administered is therefore also key to dictating the free radical activity as fullerenes exposed at high concentrations are more likely to interact to form larger structures, which is known to detrimentally impact on its antioxidant behavior. Many of the studies reported have used exceptionally high exposure concentrations and are therefore difficult to interpret in terms of relevance unless large doses are used in such applications.

Uptake of Fullerenes into Cells

Uptake studies have investigated the behavior of both professional phagocytes, such as macrophages, and nonphagocytic cells. Determining the uptake of fullerenes by cells is of relevance as phagocytic cells, located at exposure sites, are responsible for the clearance of particles. Second, the uptake of fullerenes by cells has the potential to impact on normal cell physiology and function, which requires assessment. When addressing the uptake of fullerenes, a variety of cell types have been considered. In addition, computer simulations have been conducted that act to predict the interactions of fullerenes with cell membranes and their subsequent penetration.

It has been observed that subsequent to pulmonary exposure, fullerenes are evident within alveolar macrophages (Fujita *et al*., 2009; Xia *et al*., 2006). Furthermore, subsequent to ip administration, fullerenes enter the circulation and have been observed to accumulate within Kupffer cells in the liver (Gharbi *et al*., 2005). Macrophages therefore appear to be capable of taking up particles to thereby fulfill their role within host defense. However, the consequences of fullerene uptake

require attention as oxidative or inflammatory events may be stimulated. In addition, a number of other cell types have been demonstrated to internalize fullerenes, such as keratinocytes (Rouse *et al*. 2006), epithelial cells (Fujita *et al*., 2009), and eye lens cells (Roberts *et al*., 2008) often with oxidative and lethal consequences.

Computer simulation has been exploited to reveal the mechanism of fullerene permeation through cell membranes (Wong-Ekkabut *et al*., 2008). It was identified that small fullerene clusters (< 10 molecules) were able to localize within the membrane lipid bilayer, where they disaggregated, that this process was passive and spontaneous, and that even at high concentrations, no mechanical damage to the membrane was observed. However, although the computer simulations provide insight into the possible behavior of fullerenes, caution is required when extrapolating the results as the interactions of fullerenes with other membrane components, such as other lipids, carbohydrates, and proteins, were not considered within the model, and it is therefore necessary to verify the results. However, the tendency for fullerenes to interact with the lipid tails within the model parallels the finding that lipid peroxidation is a prominent feature of their exposure to cells.

In addition, Bedrov *et al*. (2008) used molecular dynamic models to investigate the interaction and transport of C_{60} within a plasma membrane model. C_{60} was observed to interact with the lipid head groups and lipid core of the membrane. Consequently, fullerenes were predicted to have a high permeability within the simulations conducted. For this reason, the authors suggested that fullerenes could be exploited as efficient carriers to enable drug entry into cells. Similarly, Qiao *et al*. (2007) used molecular dynamics to study the translocation of fullerenes across a model cell membrane. Pristine C_{60} was observed to readily translocate across the lipid membrane. This was achieved due to the ability of C_{60} to create a cavity (termed transient micropores) within the membrane. C_{60} molecules were then speculated to "jump" into the membrane, which enabled the penetration of the molecule through the membrane. In contrast, $C_{60}(OH)_{20}$ derivatives barely penetrated the membrane, which was explained by their absorption onto the membrane surface, due to its hydrophilic nature. This interaction discouraged $C_{60}(OH)_{20}$ interaction with the lipid core of the membrane so that it did not enter the membrane, but instead, the strong interactions with the membrane surface head groups caused a "pinch" to form in the plasma membrane. Therefore, pristine fullerenes and fullerene derivatives exhibit differences, with regards to their translocation through the model membrane, and perhaps this can explain differences in their toxicity and why fullerene derivatives exhibit reduced toxicity than their pristine counterparts (see, e.g., Sayes *et al*., 2004). However, mainly, the simulations did not consider the aggregation state of fullerenes and depict the consequences of membrane exposure to individual molecules, which would be anticipated to more easily enter cells due to their small size (< 1 nm).

Porter *et al*. (2006) found that no cytotoxicity to human-derived macrophages was elicited by C_{60} (0.16–10 μg/ml, prepared using THF and dispersed in serum-free cell culture medium) *in vitro*, despite the fact that they were internalized and contained within the cytoplasm, nucleus, and lysosomes. It was observed that C_{60} crystals and aggregates were contained within lysosomes (perhaps as a consequence of their internalization by phagocytosis or endocytosis) in which C_{60} was degraded into smaller structures. C_{60} aggregates were also apparent along the plasma membrane, which was suggested by the authors to promote the development of lipid peroxidation observed by other investigators. It was highlighted that there was a difficulty in imaging particles, due to the fact that it is difficult to distinguish them from artifact presence, and so the results require further verification.

The uptake of fullerenes has been demonstrated on numerous occasions within a variety of cell types. The implications of uptake are relatively unknown, and therefore worthy of consideration in the future, but are likely to involve oxidative or cytotoxic responses. Computer simulations have also been used to predict the penetration of fullerenes within the plasma membrane and attributes of particles that encourage such an interaction, but their relevance requires confirmation.

Genotoxicity of Fullerenes

Genotoxicity tests are conducted to reveal damage to DNA elicited by fullerene exposure by, e.g., detecting mutations and changes in chromosome structure or number. A number of assays can be adopted to detect genotoxicity, including the comet assay, Ames test, and determining tumor development within animals.

Dhawan *et al*. (2006) investigated whether C_{60} was able to inflict DNA damage within human lymphocytes and was detected using the comet assay when exposed at concentrations ranging from 0.42 to 2100 μg/l (dispersed in serum-free cell culture medium) for up to 6 h. To ensure that residual solvents used to prepare C_{60} suspensions were not responsible for any observed toxicity (see below), the experiments were conducted using preparation methods that were free of organic solvents. These included prolonged mixing of C_{60} in water ($aqu C_{60}$) or the "solvent to water exchange" method using ethanol ($EtOH C_{60}$). It was demonstrated that solvents were more effective at dispersing C_{60}, as demonstrated by the fact that the size of C_{60} clusters was smaller (122 nm diameter) than those produced within $aqu C_{60}$ suspensions (178 nm diameter). Both samples were able to cause DNA damage within lymphocytes, with $aqu C_{60}$ being more effective. The results therefore highlight that the dispersion method is able to impact on the toxicity of C_{60}, whereby fullerenes prepared by mixing in water were more capable of eliciting a genotoxic response than those produced using the solvent to water exchange method. In addition, Sera *et al*. (1996) investigated the mutagenic effect of fullerene exposure (up to 30 μg/plate for 48 h) on *Salmonella typhimurium* in light and dark conditions using the Ames test. If

exposure occurred within the dark, no mutagenic responses were evident. In contrast, a mutagenic effect was observed when exposure occurred in the presence of visible light due to the production of ROS, which interact with DNA to elicit damage, and was typified by the formation of 8-hydroxydeoxyguanosine. Lipid peroxidation was also increased by fullerene exposure in light, further highlighting the oxidative consequences associated with light irradiation. The study therefore illustrated the phototoxic and mutagenic properties of fullerenes.

Contrary to the ability of C_{60} to induce genotoxic events within cells, fullerene derivatives have been demonstrated to have potential therapeutic properties for the treatment of cancer. Chen *et al*. (2005) sc implanted H22 hepatoma cells into mice, and tumor growth was monitored until the tumor that developed reached a size of 2 or 2.2 cm. Tumor-bearing mice were then treated, once a day, with $Gd@C_{82}(OH)_{22}$ (dispersed in saline), via ip injection, at a concentration of 114 or 228 μg/ml, and the antitumor effect was evaluated by determining the impact of treatment on tumor size. It was observed that $Gd@C_{82}(OH)_{22}$ inhibited skin tumor growth within hepatoma-implanted mice, with this inhibitory effect apparent on treatment of mice for 6 days or more. The antitumor efficiency of $Gd@C_{82}(OH)_{22}$ was greater than that of the conventional antineoplastic agents cyclophosphamide and cisplatin. The administration of C_{60} was associated with very low toxicity *in vivo*, and no cytotoxicity was associated with hepatocyte cell line exposure *in vitro*. Furthermore, Tabata *et al*. (1997) aimed to target radiolabeled ^{125}I C_{60}-polyethylene glycol (PEG) conjugates (424 μg/kg, dispersed in PBS) to tumors following iv injection within tumor-bearing mice. The distribution of the fullerene was determined following exposure, and 78% of the administered dose was eliminated from the body within 24 h. No marked accumulation within a particular organ was observed (probably as a consequence of its derivatization), although localization within the liver and gastrointestinal tract was observed, with no toxicity associated with exposure. The fullerene was able to accumulate within tumors due to its large size (which also relied on the hyperpermeability of tumor vasculature). Light irradiation (to achieve photoactivation of the fullerene) at the tumor site allowed the specific destruction of the tumor by the fullerenes, with no damage to the overlying normal skin. Tumor destruction was not apparent with C_{60}-PEG administration alone; therefore, light irradiation was essential for the tumor-destructive effect to manifest. Accordingly, the phototoxic property of fullerenes was exploited within the destruction of tumors, and fullerenes may therefore be considered for photodynamic therapy of tumors. However, its exploitation is reliant on the utilization of particular C_{60} derivatives; accordingly, PEG conjugation to C_{60} was necessary to increase the size of the molecule and to increase its water solubility. In addition, Zhu *et al*. (2008) evaluated the tumor inhibitory effect of fullerols (0.2 and 1 mg/kg, dispersed in deionized water, administered daily via ip injection, for up to 17 days) within the mouse H22 hepatocarcinoma model. $C_{60}(OH)_x$ exhibited antitumor activity, with treatment more effective if administered from the time of tumor inoculation onward, as opposed to when treatment was initiated after tumor growth reached 1 cm.

Furthermore, genotoxicity has not been associated with fullerene exposure in a number of models. Mori *et al*. (2006) investigated the mutagenicity of a C_{60}/C_{70} mixture. It was illustrated that no mutagenic responses were evident within a variety of *S. typhimurium* and *E. coli* strains using the Ames test (up to 5000 μg/plate). In addition, within the chromosomal aberration test (in CHL/IU hamster lung cells), no aberrations within the structure or number of chromosomes were apparent. The separate tests therefore reached the same conclusion that the fullerene investigated was not mutagenic. Furthermore, Jacobsen *et al*. (2008) investigated the mutagenicity associated with a number of carbon-based NPs, including C_{60} within the mouse FE1-Muta epithelial cell line. It was demonstrated that exposure of cells to C_{60} (dispersed in serum-containing cell culture medium, with sonication used to minimize particle aggregation) was associated with a slight increase in ROS production in cells, but no impact on cell viability was observed. This was demonstrated following a short term exposure scenario (24 h exposure, at concentrations of 20 to 200 μg/ml), and in the long term (following 9 rounds of particle exposures of 72 h for each round (total duration of 576 h), at a concentration of 100 μg/ml). An increase of ROS production by C_{60} was also observed in cell-free conditions.

The genotoxicity of fullerenes is therefore difficult to interpret from the studies conducted so far, with contradicting results reported. Again, these are likely to be influenced by the dose, dispersion, model, and end point measured. An important component of the genotoxic response exhibited by fullerenes is anticipated to be their photoactivity, which is able to promote such a response. However, the opportunity to exploit fullerenes as antitumor agents is of interest and warrants further investigation.

Reproductive Toxicology of Fullerenes

Evaluation of fullerene effects on the reproductive system is limited to a small number of *in vivo* and *in vitro* studies. Most of these have focused on how treatment can affect the developing embryo. A study by Tsuchiya *et al*. (1996) ip administered polyvinylpyrrolidone-solubilized C_{60} (up to 137 mg/kg in distilled water) to pregnant mice on day 10 (vaginal plug: day 0). After 18 h of exposure, all the embryos were examined and found to be dead. A variety of doses were examined with C_{60} apparently distributed into the embryos at 50 mg/kg resulting in head region and tail abnormalities. The yolk sac appeared with shrunken membrane and narrow blood vessels, which may suggest insufficient blood supply to the embryos. At 25 mg/kg, one embryo had abnormal enlargement of the head, whereas all other embryos appeared normal. The no-observed-adverse-effect level was 16.7 mg/kg. The study recommends further work due to the low number of animals

per exposure group. This is an unusual route of administration using a relatively high exposure dose, and it covers only a small part of the pregnancy period.

A study by Zhu *et al.* (2008) assessed embryonic development using the zebrafish model. The group exposed newly fertilized eggs to 50 mg/l fullerol ($C_{60}(OH)_{16–18}$), nano aggregates of C_{60} (1.5 mg/l, particle size: ~100 nm), or a combined treatment of *nano*C_{60} (1.5 mg/l) and GSH (30 mg/l) for 96 h. Observed end points were survival, hatching rate, heart beat rate, and pericardial edema. The fullerol solution had no adverse effect on the end points, whereas the C_{60} suspension had a conspicuous adverse effect on all parameters that was lessened by the addition of GSH. Zhu *et al.* (2008) concluded that the adverse effects of C_{60} were due, at least partly, to free radical–induced mechanism or another form of oxidative stress. Similarly, Usenko *et al.* (2008) also used zebrafish embryos to examine the potential of C_{60} to elicit oxidative stress responses. The group's findings showed reduced light, and therefore, reduced photocatalytic activity during C_{60} exposure significantly decreased mortality and the incidence of fin malformations and pericardial edema at 200 and 300 ppb (equivalent to 0.2 and 0.3 μg/ml, respectively) C_{60} 24-h postfertilization. Embryos co-exposed to the glutathione precursor, NAC, also showed reduced mortality and pericardial edema; however, fin malformations were not reduced.

The reviewed literature examines the effects of fullerenes during pregnancy, highlight effects on developing embryos; however, studies are extremely limited in number and in sample size. No specific *in vitro* or *in vivo* studies were found examining fullerene effects in male reproductive system.

Interactions of Fullerenes with Biological Molecules

The ability of fullerenes to interact with biological molecules, such as proteins, is of concern as it not only has the propensity to alter the normal structure and function of these biological moieties and enable fullerene transport within the body (as interactions of fullerenes with serum proteins are evident) but also is potentially able to modify the behavior of the particles.

Belgorodsky *et al.* (2006) demonstrated that cyclodextrin-capped fullerenes were able to form stable complexes with bovine serum albumin (BSA). The implications of fullerene binding are uncertain, but normal protein conformation and function were retained, and therefore, such an interaction is likely to facilitate fullerene transport within the body. Benyamini *et al.* (2006) used computational models to understand the interactions between fullerenes (in a pristine and carboxylated form) and proteins (namely human serum albumin, BSA, human immunodeficiency virus [HIV] protease, and a fullerene-specific antibody) that had all been previously demonstrated to interact with fullerene molecules. The binding sites for fullerenes within the proteins were identified and demonstrated to have similarities between the different proteins (although not for the fullerene antibody) investigated.

The ability of fullerenes to interact with proteins may be beneficial within the treatment of disease. Schinazi *et al.* (1993) illustrated that water-soluble fullerene derivatives exhibited antiviral activity against HIV within infected lymphocytes (with no cytotoxicity associated with the response). This antiviral activity is anticipated to derive from a direct interaction between the fullerene and the active site of the HIV protease, which causes the subsequent inhibition of enzyme activity (Friedman *et al.*, 1993). However, the design of the fullerene molecule is of vital importance to its interactions with proteins as within the described studies, the fullerene derivatives used were designed in such a way to encourage such interactions, and so the applicability of the response to fullerenes as a whole requires investigation.

The described studies highlight that fullerenes are able to interact with proteins, which may have detrimental implications for protein function. This property can be considered beneficial within their exploitation within antiviral therapy. However, the specificity of the response requires consideration as unregulated enzyme inhibition would be detrimental and likely to be associated with adverse responses. The binding of fullerenes to serum proteins is of interest as it is likely to facilitate their transport within the body to enable them to reach target sites distal to their exposure site.

LINKING THE PHYSICOCHEMICAL ATTRIBUTES OF FULLERENES TO PATHOGENICITY OR TOXICITY

As for other particles, the physicochemical properties of fullerenes are likely to impact on their toxicity. Such characteristics include composition, crystal, or aggregate size, water solubility, and surface modifications/functionalization.

Surface Modifications

Surface modification is also termed derivatization or functionalization and is used to achieve a specific change in particle properties. Common examples of particle functionalization include hydroxylation (–OH) and carboxylation (–COOH). In general, the surface of fullerenes is completed for a specific purpose, such as improving water solubility. In addition, it is recognized that such changes may also influence toxicity as, e.g., fullerene functionalization has been observed to promote the appearance of beneficial properties, such as antioxidant or anti-inflammatory activity (see above).

Accordingly, pristine (unmodified) C_{60} has been demonstrated to be more toxic than the functionalized water-soluble counterparts (including $C_{60}(OH)_{24}$) when examined *in vitro* with keratinocyte and hepatocyte cell lines (Sayes *et al.*, 2004). In fact, as the fullerene cage became more derivatized, and therefore more water soluble, the corresponding toxicity was observed to decrease. However, in a separate study, Sayes *et al.* (2007) exposed rats via intratracheal instillation (0.2–3 mg/kg, dispersed in MilliQ ultrapure water) to C_{60} or

TABLE 3
The Impact of Fullerene Dispersion on Fullerene Toxicity

Paper	Fullerene dispersion	Model	End points	Findings
Isakovic *et al.* (2006)	γ-irradiated and nonirradiated *THF*C_{60} samples	Mammalian cells (L292 [fibroblasts], B16 [melanoma], C6 [glioma], and U251 [glioma] cell lines, primary rat astrocytes, and rat and mouse macrophages)	Cytotoxicity (LDH release and apoptosis/necrosis) Oxidative stress (ROS generation, lipid peroxidation (MDA), and NAC pretreatment) ERK activation	Irradiation inactivates the *in vitro* cytotoxicity of C_{60} (which has been solubilized in THF) due an impaired capacity of C_{60} to generate free radicals (possibly due to THF decomposition)
Lyon *et al.* (2006)	*THF*C_{60}, sonicated C_{60}, *aqu*C_{60} (extended mixing in water), and polyvinylpyrrolidone prepared C_{60}	Bacteria *Bacillus subtilis*	Sample stability (TEM and dynamic light scattering) Antibacterial effect Oxidative stress (lipid peroxidation)	*Son*C_{60} and *PVP*C_{60} more uniformly dispersed, with smaller aggregate size than *THF*C_{60} and *aqu*C_{60}. All samples exhibit similar antibacterial activity Smaller aggregates (obtained via centrifugation of samples) of *THF*C_{60} and *aqu*C_{60} samples exhibit more toxicity
Markovic *et al.* (2007)	*THF*C_{60}, *EtOH*C_{60}, and *aqu*C_{60} (extended mixing in water)	Cell lines: NTCC 2544 (keratinocytes), L929 (fibroblast), and B16 (melanoma)	Cytotoxicity (MTT, apoptosis/necrosis, and morphology) Mitochondrial depolarization ROS generation (DCFH and EPR)	*THF*C_{60} caused the greatest cytotoxic effect that was not cell, assay, or species specific Cytotoxicity mediated by ROS generation THF proposed to be intercalated into C_{60} structure
Zhang *et al.* (2009)	*THF*C_{60} (washed and unwashed samples) and *Son*C_{60}	*Escherichia coli*	Oxidative reactivity (indigo dye assay) Antibacterial activity	C_{60} derivatives (THF peroxide) are responsible for antibacterial response Washing of the *THF*C_{60} sample can effectively remove the derivatives that are responsible for the observed toxicity

Note. DCFH, dichlorofluorescin; EPR, electron paramagnetic resonance; ERK, extracellular signal-regulated kinase; MDA, malondialdehyde; MTT, 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide; TEM, transmission electron microscopy.

$C_{60}(OH)_{24}$ and found that both fullerene types did not induce toxicity from 1-day to 3-month postexposure, as indicated by LDH release, neutrophil infiltration, oxidative stress, or histopathological analysis. Although none of the fullerene derivatives inflicted toxicity, the administration of quartz as a control produced a proinflammatory and profibrotic response. Therefore, although functionalization may affect *in vitro* toxicity, this may not translate *in vivo* effects, highlighting the potential difficulty in extrapolating and interpreting experimental findings (Sayes *et al.*, 2007). However, there is also a difference between the *in vitro* models and the *in vivo* models with respect to target organ under investigation; the *in vivo* study investigated pulmonary responses, while the *in vitro* cell lines included keratinocytes and hepatocytes. Therefore, fullerenes may not be universally toxic to different tissue or cell targets. In addition, it is also necessary to determine if particles are able to penetrate through the skin or become systemically available to thereby reach the different cell types studied within the *in vitro* studies.

In contrast to the results of Sayes *et al.* (2007), Kamat *et al.* (2000) demonstrated that $C_{60}(OH)_{18}$ was more toxic than underivatized C_{60} *in vitro* (see above), although both were capable of eliciting oxidative damage. Therefore, the influence of surface attachments on fullerene toxicity may be dependent upon the target cell/organ and/or the fullerene type under investigation.

The Impact of Fullerene Dispersion on Toxicity

C_{60} has a hydrophobic character, and therefore, unless rendered water soluble through derivatization of its surface (see above), a stable suspension of C_{60} in aqueous solutions is difficult to achieve (Dhawan *et al.*, 2006). However, pristine C_{60} molecules are able to form a stable water-soluble suspension that is composed of C_{60} clusters or aggregates (termed *nano*C_{60}, *n*C_{60}, or colloidal fullerenes) that generally range from 5 to 500 nm in diameter (Dhawan *et al.*, 2006; Fortner *et al.*, 2005). To obtain *n*C_{60} suspensions, a number of techniques can be employed (Dhawan *et al.*, 2006). A solvent to water exchange protocol can be followed whereby fullerenes

are mixed with a solvent (such as THF), which is then removed via evaporation or distillation. Alternatively, fullerenes can be dispersed in a solvent (such as toluene or ethanol) mixed with water and then sonicated to transfer the fullerenes to the water phase. In addition, fullerenes can be mixed with water for an extended period, which is often termed aqueous C_{60} (or *aqu*C_{60}). It has been suggested that the toxicity associated with *n*C_{60} exposure may derive as a consequence of the presence of residual solvent (or their derivatives), which have the potential to become intercalated into the lattice C_{60} structure or are released into the aqueous phase. This has implications for fullerene preparation as such impurities may impact upon the observed toxicity. A summary of the impact of the dispersal method on C_{60} toxicity is demonstrated within Table 3.

Isakovic *et al.* (2006) dispersed C_{60} using THF (termed *THF*C_{60}) and subjected the suspension to γ irradiation in order to decompose residual THF. The nonirradiated *THF*C_{60} sample (0.125–1 μg/ml in serum-containing cell culture medium) elicited cell death, via a necrotic mechanism, within all cell types tested (mouse fibroblast L929, mouse B6 melanoma cells, rat glioma C6, human glioma U251 cell lines, primary rat astrocytes, and mouse and rat macrophages). The authors concluded that this cytotoxic response was associated with increased ROS production as the toxicity was associated with lipid peroxidation, and pretreatment with the antioxidant NAC prevented the *THF*C_{60}-induced toxicity. In contrast, the irradiated *THF*C_{60} sample failed to elicit a cytotoxic effect and was instead deemed to be cytoprotective due to its antioxidant properties that were demonstrated by its ability to prevent against hydrogen peroxide–mediated toxicity in cells.

Markovic *et al.* (2007) compared the toxicity of *n*C_{60} when prepared within different solvents (THF or ethanol) or within an aqueous solution (*aqu*C_{60}) when dispersed in serum-containing cell culture medium. All fullerene preparations contained particles that had a diameter of less than 36 nm. Cytotoxicity, ROS production, and mitochondrial depolarization were evident (within human NTCC 2544 keratinocyte, human NHDF dermal fibroblast, mouse B6 melanoma, and mouse L929 fibrosarcoma cell lines), with *THF*C_{60} exhibiting the greatest toxicity and *aqu*C_{60} the least. No species, cell type, or assay dependence was observed to influence the toxicity. It was also proposed, by the authors, that THF was able to intercalate into the structure of fullerenes to increase its toxic potential.

Zhang *et al.* (2009) generated *THF*C_{60}, and the samples either remained unwashed or were washed with water in order to investigate whether residual solvent presence contributed to any observed toxicity. In addition, a *nano*C_{60} suspension was generated by first preparing C_{60} within toluene (termed *son*C_{60}). The major THF derivative contained within the *THF*C_{60} suspension was THF peroxide, which accumulated within the water phase of the sample and was introduced during the preparation of *THF*C_{60} suspensions. Washing of the sample was successful in effectively removing THF peroxide. Washed *THF*C_{60} and *son*C_{60} did not exhibit oxidative properties, but unwashed *THF*C_{60} increased ROS production, and the THF derivative was held responsible for this effect. Unwashed *THF*C_{60} was the only sample that had a bactericidal action to *E. coli* (when dispersed in Minimal Davis media), and again, its THF peroxide content was suggested to be accountable for this effect.

Fortner *et al.* (2005) investigated whether the size and stability of *n*C_{60} suspensions are related to the conditions surrounding their formation, namely the rate of water addition and pH of the solution. It was found that a higher pH promotes the formation of smaller C_{60} aggregates. In addition, by slowing the rate of water addition when conducting solvent to water exchange, the average particle size increases. These are important observations as fullerene aggregation may influence toxicity (see below).

Lyon *et al.* (2006) prepared four different preparations of *n*C_{60}, namely *THF*C_{60}, *son*C_{60}, *aqu*C_{60}, and polyvinylpyrrolidone-functionalized C_{60} (*PVP*C_{60}), and determined if the size and morphology of the aggregates were able to impact on their bactericidal behavior. The method of preparation was observed to impact on the size of the aggregates so that particles ranged from 50 to 150 nm for *THF*C_{60} and from 30to 100 nm for *aqu*C_{60}, with the *son*C_{60} and *PVP*C_{60} samples more uniformly dispersed (10–25 nm diameter). *THF*C_{60} displayed the most potent antibacterial behavior, although all samples were capable (all samples were dispersed in Minimal Davis media, which has a low potassium concentration, to reduce particle aggregation). It was also demonstrated that the solvent "controls" (i.e., contained no C_{60}) exhibited no evidence of toxicity. In addition, the *THF*C_{60}, and *aqu*C_{60} samples were centrifuged to separate larger and smaller aggregates from the suspension, and it was apparent that suspensions containing smaller aggregates had a greater propensity to display antibacterial effects. It is difficult to draw a firm conclusion from this study as the solvent for dispersion is altered in order to influence the particle size. It is therefore not clear whether the toxicity differences between samples might be due to solvent or aggregate size differences.

In general, the findings suggested that *n*C_{60} preparations are more toxic when prepared using a solvent to water exchange method, in comparison to those prepared through the extending stirring in water. Together, these studies demonstrate that trace contamination with THF is sufficient to significantly increase the toxicity of *n*C_{60}. One explanation appears to be the formation of reactive species due to interaction between the THF and C_{60}; these studies therefore highlight the need to stipulate the method of fullerene dispersion.

Therefore, within the described studies, it is often difficult to decipher whether the fullerenes themselves, or the solvents (or their derivatives) used within the preparation of fullerene suspensions, are responsible for the observed effects. Zhang *et al.* (2009) illustrated that THF solvent derivatives, which were generated during the preparation of a fullerene suspension, were accountable for observed toxicity. Similarly, solvent (DMSO) decomposition has also been held responsible for the toxicity exhibited by fullerenes within zebrafish (Henry *et al.*, 2007). Therefore, the conversion of solvents into toxic

byproducts during the particle suspension preparation is possible and potentially responsible for driving the observed toxicity of fullerenes. As such, it would be relevant to detect the formation of such solvent decomposition products within particle suspensions or include appropriate controls (such as solvent degradation products) within investigations. Therefore, it is plausible that fullerenes may be mistakenly identified as driving any observed toxicity when it is really the use of solvents within the preparation of particle suspensions that are responsible for the response observed. As such, it is necessary for investigators to consider how the preparation of fullerenes into a suspension impacts on the observed toxicity. In addition, it is of importance to consider the relevance of the preparation method used to generate particle suspensions and in particular if humans will actually encounter fullerenes in this form.

Contribution of Photocatalytic Behavior of Fullerenes to Their Toxicity

It is acknowledged that fullerenes are able to absorb ultraviolet or visible light to stimulate the generation of ROS and to ultimately increase their toxic potency (Nielsen *et al*., 2008) through the promotion of oxidative-mediated damage. The contribution of photocatalytic reactions to fullerene toxicity has been a focus of a number of previously discussed studies (Dhawan *et al*., 2006; Kamat *et al*., 2000; Roberts *et al*., 2008; Usenko *et al*., 2008). This phenomenon has also been exploited within the destruction of tumors in mice (Chen *et al*., 2005), but more work is required to determine the specificity of the response and how this may be exploited within the treatment of humans.

COMMENTS ON THE EXPERIMENTAL DESIGNS

Relevant fullerene exposure concentrations to be used within *in vitro* and *in vivo* experiments cannot be obtained without information regarding the human exposure levels, which is information that is currently lacking. However, some concentrations used have been exceptionally high and unlikely to be encountered by humans; for example, Gharbi *et al.* (2005) exposed rats to fullerenes at a dose of up to 2 g/kg via ip exposure. In addition, it is often the case that a single dose has been administered to animals or cells and toxicity assessed at a number of postexposure time points, but it is likely that the utilization of fullerenes within occupational or consumer settings will involve the exposure of humans for extended periods, and this should perhaps be considered within future studies. The uncertainty regarding human exposure levels to fullerenes also makes interpretation of the observed hazards difficult.

In addition, the most relevant way of expressing fullerene concentrations is debatable and within the studies outlined has been expressed as parts per million, micromolars, milligrams per kilogram, milligrams per milliliter, etc (which in some circumstances is likely to derive from the exposure scenario). In order to counteract this problem, molar concentrations stipulated within the described studies have been converted to a standardized dose descriptor (of mass weight of particles administered per animal, milliliters [of particle suspension], or kilograms [of the exposed animal]) in order to allow for easier comparisons between investigations. Importantly, when the molecular formula of the fullerene in question was not explicitly stated by investigators, calculations were based on the use of C_{60}. The toxicity of NPs has been demonstrated in a number of studies to be related to their small size and therefore high surface area (Brown *et al*., 2001; Duffin *et al*., 2002, 2007; Stoeger *et al*., 2006). The high propensity for fullerenes to aggregate means that not all the potential calculated surface will be available at the surface, and therefore, expressing the concentration as the surface area of particles administered is perhaps not the best dose descriptor to use for fullerenes. However, introducing a standard way of expressing fullerene concentration would also allow the opportunity to more easily compare the findings of different investigators. In line with this, it would be of benefit for investigators to provide as much information as possible, relating to the exposure conditions and particle dimensions used (including mass of particles administered, in what volume they were administered [if relevant to the experiment], the size and surface area of particles used, and the molecular formula of the fullerene) so that the concentration used can be more easily converted by others to allow comparisons between different investigations to be made, until such a decision on how to best standardize the concentration is agreed on.

There are a diverse number of fullerenes available, which derives from the number of carbon atoms contained within the cage structure, the surface modification, and the preparation processes utilized to promote their water solubility. Therefore, fullerenes used by different investigators are likely to behave differently, even if described as *THF*C_{60}, *aqu*C_{60}, etc as the processes underlying their generation by different groups will be distinct. Full physicochemical characterization is therefore essential to allow comparisons between fullerene toxicity to be made. This is an important issue as the physicochemical characterization of fullerenes (and nanomaterials as a whole) is often lacking within the published literature. It is relevant to highlight that a series of reviews relating to the toxicity of four nanomaterial types, namely carbon nanotubes, metal oxides, metals, and fullerenes, were considered within the ENRHES project and that fullerenes were the least extensively characterized material studied. As such, it is recommended that the particle size, surface area, aggregation tendency, composition, surface chemistry, charge, crystal structure, and solubility are determined by investigators when identifying the hazards associated with nanomaterial exposure (see Fig. 1). This is in line with the recommendations of Warheit (2008) who highlighted which parameters should be assessed with the highest priority when conducting the physicochemical analysis of nanomaterials prior to the initiation of toxicological studies. However, a number of different methods can be utilized to

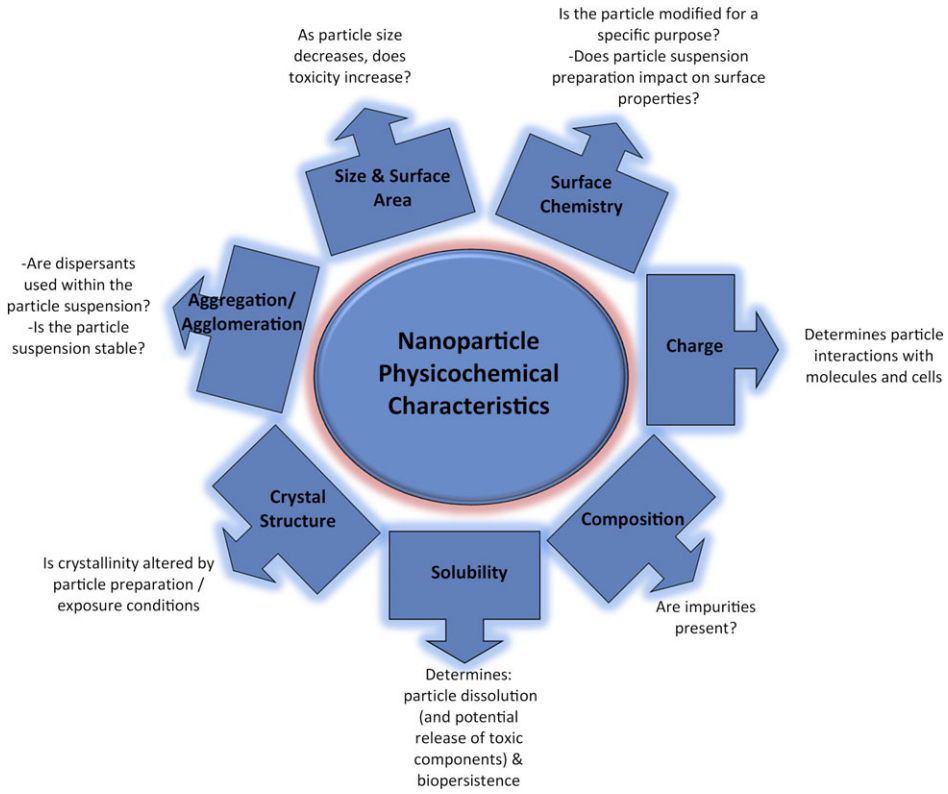

FIG. 1. Physicochemical characterization of nanomaterials. Physicochemical properties of nanomaterials that should be considered with high priority in toxicological assessments are outlined. Determining the size, surface area, aggregation/agglomeration tendency, composition, surface chemistry, charge, crystal structure, and solubility of the nanomaterial under investigation is therefore critical, as these attributes have been consistently demonstrated to influence the toxicity of nanomaterials.

measure one of these end points; for example, to determine particle size, electron microscopy, dynamic light scattering, and atomic force microscopy can all be conducted, and each is likely to provide different results. Therefore, assessing which technique is most appropriate and relevant to use is challenging. In addition, the situation is complicated by the fact that the experimental design has the potential to impact on the physicochemical characteristics of nanomaterials and as a consequence their toxicity by impacting, e.g., on aggregation tendency or surface chemistry. Therefore, it is necessary for investigators to explicitly state what particle preparation methods and exposure conditions were used when assessing the toxicity of nanomaterials. This will allow for the easier identification of which properties of particles drive the observed responses. Accordingly, the fact that general conclusions cannot be made regarding fullerene behavior and toxicity is logical and expected. Toxicity may be driven by the fullerene under investigation as the toxicity of C_{60} has been demonstrated to not be assay, cell and species specific (Markovic *et al*., 2007). However, this is based from the findings of one study, and therefore, this finding requires further investigation. So far, there is evidence of a lack of correlation between *in vitro* and *in vivo* studies (Sayes *et al*., 2007), although this can in part be explained by the differences in cell targets studied between the *in vitro* study (Sayes *et al*., 2004) and the *in vivo* model (rat lung, Sayes *et al*., 2007).

CONCLUSIONS

There are a number of factors that appear to be implicated in fullerene behavior and toxicity, including chemical structure, surface modifications, and preparation procedure. Ultimately, these factors drive fullerene water solubility, which appears to be related to antioxidant/cytoprotective or pro-oxidant/cytotoxic properties. Generally, the greater the water solubility exhibited by a fullerene sample, the lesser the toxicity associated with exposure. However, the situation is complicated by the findings that residual solvents (or their derivatives) used within the preparation of fullerene samples are able to contribute to the observed toxicity, which negates improving water solubility by particular methods. In addition, the preparation of surface-modified fullerenes is conducted to improve an aspect of fullerene function and also modifies the toxicity of fullerenes. However, the fact that fullerene derivatives do not always behave similarly to their unmodified counterparts is expected and may allow for the safe integration of fullerenes into products by revealing which

attributes of particles are most influential in driving toxicological findings.

The studies conducted so far suggest that fullerene toxicity involves an oxidant-driven response, suggesting that toxicity evaluations should evaluate the potential of fullerenes to cause oxidative stress and related consequences, such as inflammation or genotoxicity. The studies conducted with fullerenes thus far are rather limited in terms of models used, targets investigated, and mechanisms of toxicity. Much more work is required to generate sufficient knowledge to inform a risk assessment. Accordingly, it is unrealistic to make generalizations about the behavior of fullerenes from the limited number of studies that have been conducted as investigations into the toxicity of fullerenes via specific routes of delivery, or at particular cell and organ targets, are often too few in number to make definite conclusions about fullerene behavior. In addition, the quality (including the concentrations used and experimental model) of conducted experiments is relevant to consider, which is of vital importance when considering the risk associated with fullerene exposure.

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