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REVIEW ARTICLE

Toxicity of pristine versus functionalized fullerenes: mechanisms of cell damage and the role of oxidative stress

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Abstract The fullerene C_{60}, due to the physicochemical properties of its spherical cage-like molecule build exclusively from carbon atoms, is able to both scavenge and generate reactive oxygen species. While this unique dual property could be exploited in biomedicine, the low water solubility of C_{60} hampers the investigation of its behavior in biological systems. The C_{60} can be brought into water by solvent extraction, by complexation with surfactants/polymers, or by long-term stirring, yielding pristine (unmodified) fullerene suspensions. On the other hand, a modification of the C_{60} core by the attachment of various functional groups results in the formation of water-soluble fullerene derivatives. Assessment of toxicity associated with C_{60} preparations is of pivotal importance for their biomedical application as cytoprotective (antioxidant), cytotoxic (anticancer), or drug delivery agents. Moreover, the widespread industrial utilization of fullerenes may also have implications for human health. However, the alterations in physicochemical properties imposed by the utilization of different methods for C_{60} solubilization profoundly influence toxicological effects of fullerene preparations, thus making the analysis of their potential therapeutic and environmental toxicity difficult. This review provides a comprehensive evaluation of the in vitro and in vivo toxicity of fullerenes, focusing on the comparison between pristine and derivatized C_{60} preparations and the mechanisms of their toxicity to mammalian cells and tissues.

Keywords Fullerenes · Cytotoxicity · Genotoxicity · Oxidative stress · In vitro · In vivo

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Introduction

Fullerenes, the spherical carbon cage sp^2-hybridized molecules, represent a third carbon allotrope. The C_{60} molecule, consisting of 60 carbon atoms, was the first fullerene discovered by Kroto et al. (1985) in soot derived from ablation of graphite with laser. Numerous fullerenes with other carbon numbers, for example higher fullerenes (C_{70}, C_{76}, C_{78}, C_{82}) and the lower-order fullerenes (C_{28}, C_{36}), have been subsequently produced (Diederich et al. 1991; Guo et al. 1992; Piskoti et al. 1998). Fullerenes have since been found to occur naturally in materials affected by high-energy events such as lightning strikes, in meteors and geologic samples (Buseck et al. 1992; Pizzarello et al. 2001).

The most common fullerene in natural occurrence is C_{60}, a stable icosahedron with C_5–C_5 single bonds forming 12 pentagons and C_5–C_6 double bonds forming 20 hexagons (Krätschmer et al. 1990). Thirty carbon double bonds present in the C_{60} structure readily accept free radicals, so C_{60} has been designated as a "free radical sponge" (Krusic et al. 1991). On the other hand, C_{60} is able to generate highly reactive oxygen species (ROS) after excitation by visible or UV light (Arbogast et al. 1991; Guldi and Prato 2000). Fullerenes efficiently generate singlet oxygen ($^1O_2$) upon irradiation via energy transfer to oxygen (type II photochemical mechanism) (Arbogast et al. 1991). The photoirradiation of fullerenes may also result in the

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production of radical anion ($C_{60}^{\cdot-}$) with subsequent generation of superoxide ($O_2^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$) via electron transfer, especially in the presence of reducing agents (type I photochemical mechanism) (Yamakoshi et al. 1998). This unique feature of C_{60} to either quench or generate cell-damaging ROS, altogether with its small size and high surface area, could be exploited in biomedicine (Markovic and Trajkovic 2008). However, the investigation of the biological properties of fullerene C_{60} has been hampered by its almost complete lack of solubility in polar solvents and the consequent formation of aggregates in aqueous solutions (Torres et al. 2011). The pure C_{60} can be brought into water by means of solvent extraction, by incorporation into water-soluble supramolecular structures, or simply by long-term stirring. The application of these methods results in formation of water suspensions containing fullerene molecules of unmodified structure ("pristine" fullerenes). On the other hand, covalent attachment of various groups (e.g., –OH, NH_2, –COOH) to the fullerene cage results in the formation of "functionalized" fullerene molecules that are able to establish bonds with water molecules via hydrophilic addends (Da Ros and Prato 1999). These compounds exhibit greater water solubility than pristine C_{60}, which makes them more attractive candidates for biomedical applications (Bosi et al. 2003; Partha and Conyers 2009). Moreover, functionalization of the fullerene core decreases its capacity for $^1O_2$ generation (Prat et al. 1999; Bensasson et al. 2001) while increasing ROS-quenching ability (Markovic and Trajkovic 2008), which led some researchers to assume that functionalized fullerenes might be less toxic than their pristine counterparts (Sayes et al. 2004; Markovic and Trajkovic 2008).

While the recent research has unveiled much about the physicochemical properties of C_{60}, the biological activities of fullerenes are less clear. Fullerenes were shown to display a range of biological activities potentially useful in cytoprotection, anticancer and antimicrobial therapy, DNA photocleavage, enzyme inhibition, controlled drug delivery, and contrast- or radioactivity-based diagnostic imaging (Satoh and Takayanagi 2006; Bakry et al. 2007). It has been shown that both pristine and functionalized fullerenes have protective effect in ROS-dependent experimental models of cell damage, but the toxic responses also occurred (Nielsen et al. 2008; Aschberger et al. 2010). The singlet oxygen, which can be produced by fullerenes in the type II photochemical mechanism, readily reacts with polyunsaturated fatty acids and amino acids, causing lipid peroxidation to inflict damage to cellular membranes or inactivating specific enzymes and damaging DNA (Briviba et al. 1997; Davies 2003). The superoxide anion radical, generated by fullerenes in the type II photochemical mechanism, can be converted to other harmful ROS such as hydrogen peroxide and hydroxyl radical, which can cause further cell damage or modulate intracellular signaling (Ray and Huang 2012). The toxicity of fullerenes, possibly driven by their pro-oxidant properties, could limit their potential for biomedical applications, while the rapidly developing industrial production of fullerene particles arouses the need for safety evaluations in the environmental and occupational settings (Morimoto et al. 2010a). Therefore, as emphasized in the recent reviews, a thorough analysis is required in order to assess what attributes of fullerenes are responsible for driving their toxic effects (Kolosnjaj et al. 2007; Johnston et al. 2010). We have recently reviewed the role of ROS in cytotoxic and cytoprotective effects of fullerenes, with the emphasis on the in vitro studies due to better mechanistic insights (Markovic and Trajkovic 2008). Having in mind the assumption that derivatized fullerenes might be more biocompatible than their pristine counterparts, the present study focuses on the comparison of the toxic effects of pristine versus derivatized fullerenes on mammalian cells and tissues in various in vitro and in vivo models. The toxicity of fullerenes toward non-mammalian (e.g., aquatic) organisms, as well as their cytoprotective and anticancer actions, is only briefly discussed, so the reader is directed to consult other recent reviews dealing with these subjects (Bakry et al. 2007; Mroz et al. 2007; Markovic and Trajkovic 2008; Partha and Conyers 2009; Henry et al. 2011).

In vitro toxicity of pristine and derivatized C_{60}

Both pristine and derivatized C_{60} are able to gain access to intracellular space or accumulate at the cell membrane (Foley et al. 2002; Sayes et al. 2005; Porter et al. 2006; Chirico et al. 2007; Li et al. 2008; Su et al. 2010; Zhang et al. 2011), thus posing a threat to cell functioning and integrity. The methods employed for solubilization of pristine C_{60} (solvent exchange, mechanical processing, and long-term stirring in water), as well as fullerene derivatization, affect its physicochemical properties and could influence ROS-related behavior and fullerene toxicity (Markovic and Trajkovic 2008). We will first give an overview of the C_{60} in vitro toxicity in general and then continue with the analysis of the toxic effects of pristine versus derivatized fullerenes on specific cell types. The cytotoxic effects of various C_{60} preparations on different cells are summarized in Table 1.

In vitro toxicity of pristine C_{60}

The fullerene colloid prepared by solvent exchange with tetrahydrofuran (THF/nanoC_{60}) has been shown to exert toxic effects on various mammalian cells in the absence of intentional photosensitization (Sayes et al. 2004, 2005;

Table 1 In vitro cytotoxicity of C_{60}

Fullerene type	Cells/targets	Photoexcitation	Main effects	References
THF/nanoC_{60}	Human dermal fibroblasts, liver carcinoma cells, astrocytes	No	↑ROS, lipid peroxidation, cell death	Sayes et al. (2004) Sayes et al. (2005)
	L929 fibrosarcoma, C6 glioma, U251 glioma	No	↑ROS, necrosis	Isakovic et al. (2006a, b)
	Human keratinocytes and dermal fibroblasts, L929 fibrosarcoma, B16 melanoma	No	↑ROS, mitochondrial depolarization, necrosis	Markovic et al. (2007)
	U251 glioma, C6 glioma, rat astrocytes	No	↑ROS, ↑ERK, necrosis (high doses) G_2/M cell cycle block, autophagy (low doses)	Harhaji et al. (2007)
	Chinese hamster ovary cells, canine kidney cells	No	Cell death	Han and Karim (2008)
	RAW 264.7 macrophages	No	↑ROS, ↑Ca^{2+}	Kovochich et al. (2009)
	Human red blood cells	No	↑ROS, hemolysis	Trpkovic et al. (2010)
	Human HaCaT keratinocytes	Yes	↑$^1O_2$, cell death, photo- and "dark" toxicity	Zhao et al. (2008a)
	Human keratinocytes	Yes	↓Cell growth, also in the dark	Bullard Dillard et al. (1996)
Sonicated C_{60}	Mouse renal epithelial cells	No	↓Transepithelial electrical resistance	Blazer-Yost et al. (2011)
Water-stirred C_{60}	Human umbilical vein endothelial cells	No	G_1 cell cycle block, ↑Ca^{2+}	Gelderman et al. (2008)
γ-Cyclodextrin C_{60}	Human lens epithelial cells, human HaCaT keratinocytes	Yes	↑$^1O_2$, ↑intracellular peroxides, apoptosis (phototoxicity)	Zhao et al. (2008b) Zhao et al. (2009)
	Rat liver microsomes	Yes	↑$^1O_2$, lipid peroxidation, ↓glucose-6-phosphatase and ATPase activity	Kamat et al. (1998) Kamat et al. 2000
C_{60}/PS-b-PDMA micelles	RIF-1 fibrosarcoma	Yes	$^1O_2$ generation, cell death (phototoxicity)	Metanawin et al. (2011)
$C_{60}(OH)_n$	L929 fibrosarcoma, C6 glioma, U251 glioma	No	ROS-independent apoptosis	Isakovic et al. (2006b)
$C_{60}(OH)_{22–26}$	Human lens epithelial cells	Yes	↑ROS, apoptosis (phototoxicity)	Roberts et al. (2008)
	Human retinal pigment epithelial cells	Yes	↑$^1O_2$, apoptosis, necrosis (phototoxicity)	Wielgus et al. (2010)
$C_{60}(OH)_{24}$	Human umbilical vein endothelial cells	No	Autophagy, ↓cell growth	Yamawaki and Iwai (2006)
	Human umbilical vein endothelial cells	No	G_1 cell cycle block, ↑Ca^{2+}, ↑ICAM-1, tissue factor and PS expression, apoptosis	Gelderman et al. (2008)
	RAW 264.7 macrophages	No	Foam cell–like formation, ↑LDL receptor expression, ↑MMP-9 secretion	Niwa and Iwai (2007)
	Human HaCaT keratinocytes	Yes	↑$O_2^{\cdot-}$, ↓mitochondrial activity	Zhao et al. (2008b)
$C_{60}(OH)_{32}$	Human epidermal keratinocytes	No	Cell death, ↑IL-8	Saathoff et al. (2011)

Table 1 continued

Fullerene type	Cells/targets	Photoexcitation	Main effects	References
$C_{60}(OH)_{15}(ONa)_9$	Porcine renal proximal tubule cells	No	Cytoskeleton disruption, autophagy, mitochondrial dysfunction, ↓ATP	Johnson-Lyles et al. (2010)
Malonic and methanophosphonic acid C_{60} derivatives	Human erythrocyte membranes	Yes	Lipid peroxidation	Yang et al. (2007)
Tris-malonyl-C_{60}	Jurkat T cells	Yes	Cell death (phototoxicity)	Rancan et al. (2002)
	Human dermal fibroblasts	No	↑ROS, cell death	Sayes et al. (2004)
C_{60} derivatives with cationic chains	Human red blood cells	No	Hemolysis	Bosi et al. (2004)
C_{60} phenylalanine derivative	Human epidermal keratinocytes	No	Cell death, ↑IL-1, IL-6, IL-8	Rouse et al. (2006)

Only the studies demonstrating the adverse effects of C_{60} are presented (for the reports on the absence of toxicity, refer to manuscript text)

ERK extracellular signal–regulated kinase, *IL* interleukin, *ICAM-1* intercellular adhesion molecule 1, *MMP-9* matrix metallopeptidase 9, *PS-b-PDMA* poly(styrene-b-dimethylacrylamide), *PS* phosphatidylserine, *ROS* reactive oxygen species, *LDL* low-density lipoprotein, $^1O_2$ singlet oxygen, $O_2^{\cdot-}$ superoxide anion radical, *THF* tetrahydrofuran

Isakovic et al. 2006a, b; Markovic et al. 2007; Harhaji et al. 2007, 2008; Han and Karim 2008; Zhao et al. 2008a; Kovochich et al. 2009; Trpkovic et al. 2010). Treatment with THF/nanoC_{60} was associated with ROS production, extracellular signal–regulated kinase activation, mitochondrial depolarization, and lipid peroxidation, eventually leading to cell membrane damage and necrotic cell death (Sayes et al. 2005; Isakovic et al. 2006a, b; Markovic et al. 2007; Harhaji et al. 2007, 2008). At subcytotoxic concentrations, THF/nanoC_{60} inhibited cell proliferation by causing autophagy, a process of self-digestion of intracellular components, associated with a G_2/M phase cell cycle arrest and the absence of necrosis or apoptosis (Harhaji et al. 2007). However, it is now recognized that the observed toxicity of THF/nanoC_{60} depends on fullerene preparation method and the properties of the solvent used (THF), rather than on intrinsic properties of the fullerene core. First, it was shown that a significant amount of THF ($\sim$10 %) remains intercalated into the fullerene crystalline lattice during nanoC_{60} preparation (Fortner et al. 2005). Furthermore, γ-irradiated THF/nanoC_{60} failed to generate ROS and cause necrotic cell death in various cell types, probably as a consequence of γ-irradiation-mediated decomposition of THF (Isakovic et al. 2006a). The C_{60} nanoparticles prepared by solvent exchange using ethanol or toluene instead of THF, as well as water-stirred C_{60}, did not produce ROS and cause significant cell damage (Markovic et al. 2007; Xia et al. 2010a). These findings favored the argument that the toxicity of THF/nanoC_{60} might be mainly due to adverse effects of residual organic solvent, although THF itself was without cytotoxic effects even at high concentrations (Isakovic et al. 2006b; Harhaji et al. 2007; Spohn et al. 2009). It was further elucidated that the toxicity of THF/nanoC_{60} actually resulted from THF degradation byproducts such as 2-hydroxytetrahydrofuranol, γ-butyrolactone, and the highly reactive THF hydroperoxide, generated by the oxygenation of THF rather than in interaction with the nanoC_{60} (Spohn et al. 2009; Henry et al. 2007; Kovochich et al. 2009). Among these breakdown products, γ-butyrolactone was shown to induce an increase in mitochondrial Ca^{2+} that was associated with a cytotoxic outcome (Kovochich et al. 2009), while THF hydroperoxide was found to account for the antibacterial activity of THF/nanoC_{60} (Zhang et al. 2009).

The mechanochemically prepared fullerene solutions comprise of particles enclosed in surfactants or other solubilizing agents (e.g., cyclodextrins or polymers), and they are able to generate ROS when photoexcited (Zhao et al. 2008a, b). The porosity of fullerene coating and the chemical properties of the surfactant/solubilizing agent might influence the mechanisms of ROS generation and cytotoxic activity. The lipid peroxidation exerted by γ-cyclodextrin/C_{60} was suppressed by the application of singlet oxygen quenchers, thus indicating involvement of $^1O_2$ (Kamat et al. 1998; Zhao et al. 2009). Cytotoxicity of water-soluble C_{60} complexes with poly(styrene-b-dimethylacrylamide) micelles also depended on $^1O_2$ generation (Metanawin et al. 2011). On the other hand, photosensitization of C_{60} complexes with polymers such as polyvinyl pyrrolidone or polyethylene glycol mainly resulted in the production of superoxide anion (Yamakoshi et al. 2003; Liu et al. 2007). In the absence of photoexcitation, fullerenes prepared with γ-cyclodextrin, anionic surfactant sodium dodecyl sulfate, or the copolymer ethylene vinyl

acetate–ethylene vinyl versatate did not exert cytotoxic activity (Misirkic et al. 2009; Trpkovic et al. 2010) and even prevented toxic effects of a highly reactive free radical nitric oxide in mammalian cells (Misirkic et al. 2009).

The C_{60} solution prepared by extended stirring in water (aqu/nanoC_{60}) was shown to yield formation of large aggregates and low fullerene concentration (Markovic and Trajkovic 2008). The aqu/nanoC_{60} prepared by sonication readily produced singlet oxygen upon illumination (Käsermann and Kempf 1997), although others found that it was not photoactive (Brunet et al. 2009, Hotze et al. 2008). In ambient light conditions, aqu/nanoC_{60} did not produce ROS and exhibited only marginal ROS-independent cytotoxicity in various mammalian cells (Markovic et al. 2007) but induced oxidative stress in chronically exposed fish (Zhu et al. 2008). The accidental release of C_{60} in the environment will potentially form aqu/nanoC_{60} upon contact with water (Duncan et al. 2007), so the assessment of C_{60} environmental toxicity needs to be further fostered (Henry et al. 2011).

In vitro toxicity of derivatized C_{60}

The degree to which the fullerene core is functionalized appears to affect the tendency of C_{60} to form aggregates. Accordingly, monofunctionalized molecules tend to aggregate more than polyfunctionalized fullerenes which exhibit greater stability (Hotze et al. 2008). It is generally believed that the derivatization of the fullerene cage significantly decreases the toxic properties of functionalized fullerenes. Accordingly, more highly soluble fullerenes have less pronounced cytotoxic activity, presumably due to the reduction in the ROS-generating capacity that occurs upon increasing the number of covalently attached functional groups (Hamano et al. 1997; Prat et al. 1999; Bensasson et al. 2001; Sayes et al. 2004). However, it has been shown that polyhydroxylated fullerenes (also termed fullerols or fullerenols) generate singlet oxygen after irradiation with UV and visible light, while superoxide production was found not only upon photoexcitation but also in the dark in the presence of reducing agents such as NADH (Pickering and Wiesner 2005; Vileno et al. 2006; Badireddy et al. 2007). Although fullerol was less efficient than polymer-coated pristine C_{60} in generating ROS (Brunet et al. 2009), it was shown to induce photooxidative stress in human cells (Vileno et al. 2006). Even at ambient light, fullerol was found to exhibit cytotoxic activity and block the cell cycle in the G_1 phase (Su et al. 2010), but this ROS-independent apoptotic cell death was observed only at fairly high doses ($LD_{50} \sim 1{,}000$ μg/ml) (Isakovic et al. 2006b). At subtoxic concentrations, however, fullerol antagonized cytotoxic action of oxidative stress–inducing agents (Dugan et al. 1996; Isakovic et al. 2006b; Harhaji et al. 2008), consistent with its ability to scavenge hydroxyl radical (Kato et al. 2009a). Similarly, carboxyfullerenes are recognized as potent antioxidative/cytoprotective agents (Dugan et al. 2001) but readily exert cytotoxicity upon photoexcitation (Rancan et al. 2002; Sayes et al. 2004). The cytotoxic properties of fullerene derivatives are also influenced by their ability to penetrate cellular membranes. Accordingly, the cationic fullerene derivatives with high hydrophobic/hydrophilic surface ratio entered the cells more easily and were markedly more toxic than neutral and anionic derivatives (Bosi et al. 2004).

In vitro cardiovascular toxicity of C_{60}

The assessment of cardiovascular toxicity is an absolute prerequisite before parenteral administration of fullerenes in their potential therapeutic and/or diagnostic applications. Nanoparticles may also translocate into the circulation after pulmonary exposure and could directly affect endothelial cells, causing vascular injury. Furthermore, the inhalation of nanoparticles may result in a release of inflammatory mediators and activation of blood cells, which can contribute to cardiovascular adverse effects (Simeonova and Erdely 2009).

The water-stirred nanoC_{60} was found to increase intracellular Ca^{2+} concentration and cause arrest in G_1 cell cycle phase in human umbilical vein endothelial cells, but no cell death was observed (Gelderman et al. 2008). In another study, sonicated C_{60} was shown to reduce endothelium-dependent vasodilatation in the aortic segments of apolipoprotein E knockout mice, implying that pristine C_{60} may disturb vasomotor balance in the presence of lipid abnormalities and atherosclerosis (Vesterdal et al. 2009). The organic solvent-prepared C_{60} grafted onto the polyurethane surface was found to efficiently adhere platelets (Lin and Wu 1999), indicating that it may promote coagulation and thrombosis. However, sonicated C_{60} was less efficient than other carbon nanoparticles in eliciting platelet aggregation, and it had no effect on the development of thrombosis (Radomski et al. 2005).

In order to assess the cardiovascular toxicity of functionalized fullerenes, it has been shown that the treatment of vascular endothelial cells with fullerol reduced the cell density and induced vacuole formation in the cytosol, associated with the accumulation of polyubiquitinated proteins and autophagic cell death (Yamawaki and Iwai 2006). In another study, fullerol was found to induce Ca^{2+} increase and G_1 cell cycle arrest in human umbilical vein endothelial cells, similar to water-stirred C_{60} (Gelderman et al. 2008). However, unlike water-stirred C_{60}, fullerol caused apoptotic cell death of vascular endothelial cells, thus possibly being able to promote atherosclerosis and coronary artery disease (Gelderman et al. 2008). In

addition, it has been shown that fullerol, together with oxidized low-density lipoprotein, could induce lipid accumulation and expression of oxidized low-density lipoprotein receptor and matrix metalloprotease-9 in cultured macrophages (Niwa and Iwai 2007), which are crucial events in atherosclerotic plaque maturation and promotion of arterial wall instability (Chen et al. 2005). Furthermore, it has been shown that fullerol facilitates ADP-induced platelet aggregation (Niwa and Iwai 2007). On the other hand, it inhibited superoxide production and protein kinase C–dependent proliferation of human coronary artery smooth muscle cells (Lu et al. 1998), which may be considered beneficial, having in mind that abnormal accumulation of smooth muscle cells and oxidative stress are the hallmarks of atherosclerosis (Ross 1999). Accordingly, hexasulfobutyl-C_{60}, another fullerene derivative with strong quenching capacity, was found to protect low-density lipoprotein from oxidation, implying its potential usefulness in atherosclerosis prevention (Lee et al. 2000).

In vitro hematological toxicity of C_{60}

The high degree of blood compatibility is required for the systemic biomedical application of fullerene compounds, particularly in light of the findings that C_{60} nanoparticles display prolonged vascular retention (Leavens et al. 2010). It has been shown that THF/nanoC_{60}, in the absence of intentional photoirradiation, causes oxidative stress–mediated lysis of human erythrocytes, associated with their shrinkage, crenation, and loss of a typical discoid shape (Trpkovic et al. 2010). The observed ROS-mediated hemolysis was apparently dependent on the residual presence of the organic solvent (THF), as C_{60} prepared by complexation with gamma-cyclodextrin or vinyl acetate–ethylene vinyl versatate did not display any significant hemolytic effect.

As for the hemolytic properties of fullerene derivatives, the ROS-mediated erythrolysis was observed after photoexcitation of dimalonic acid C_{60}, while trimalonic counterpart exhibited no significant effect (Yang et al. 2007). This is consistent with the report that the cytotoxicity of malonic acid fullerene derivatives decreases with the increase in malonic acid number added to the fullerene cage (Yang et al. 2002). On the other hand, the ROS-mediated toxicity of bis-methanophosphonate C_{60} was found to be much higher than that of mono-methanophosphonic acid C_{60}, while the hemolytic properties of fullerol were not significant (Yang et al. 2007). In the absence of overt photoirradiation, water-soluble C_{60} derivatives with polycationic adducts attached to the fullerene core were highly hemolytic, but the neutral or anionic derivatives did not cause adverse effects (Bosi et al. 2004). The observed variation in hemolytic activity might stem from the different potency of these fullerene derivatives to interact with the erythrocyte membrane.

In vitro hepatotoxicity of C_{60}

Although the fullerenes that enter the blood stream will translocate to liver, little is known about the hepatotoxic effects of fullerenes in vitro. Upon photosensitization, γ-cyclodextrin/C_{60} induced singlet oxygen–dependent oxidative stress in rat liver microsomes, causing lipid peroxidation and protein oxidation, as evidenced by the suppression of membrane-bound enzymes such as adenosine triphosphatase and glucose-6 phosphatase (Kamat et al. 1998). Similarly, polyhydroxylated fullerene caused phototoxic lipid and protein oxidation in rat liver microsomes (Kamat et al. 2000). The exposure of isolated rat hepatocytes to $C_{60}(OH)_{24}$ in ambient light conditions induced cell death associated with lipid peroxidation, mitochondrial disruption, and depletion of intracellular ATP (Nakagawa et al. 2011). The treatment with $C_{60}(OH)_{12}$ yielded less-pronounced hepatotoxic effects, thus indicating that the hepatotoxicity of fullerols may depend on the number of hydroxyl groups attached to the carbon cage (Nakagawa et al. 2011).

In vitro nephrotoxicity of C_{60}

Besides parenteral administration, the secondary renal exposure to fullerenes may be expected through lung, skin, or gastrointestinal absorption. It has been shown that THF/nanoC_{60} is toxic to canine kidney cells (Han and Karim 2008), while the exposure of mouse renal epithelial cells to sonicated C_{60} caused a significant decrease in transepithelial electrical resistance as a marker of the epithelial barrier function (Blazer-Yost et al. 2011). The polyhydroxylated fullerene was toxic to porcine renal proximal tubule cells, causing oxidative stress–independent cytoskeleton disruption and autophagic vacuole accumulation associated with mitochondrial dysfunction and subsequent ATP depletion (Johnson-Lyles et al. 2010).

In vitro ocular toxicity of C_{60}

The fullerenes may be used as drug carriers to bypass the blood ocular barriers; thus, there is a need to assess the ocular toxicity of these compounds. It has been shown that γ-cyclodextrin/C_{60} is readily internalized by human lens epithelial cells, causing a singlet oxygen–mediated protein peroxidation and apoptotic cell death upon UV irradiation, while no effect was observed at ambient light or in the dark (Zhao et al. 2009). The rate of singlet oxygen production decreased with an increased aggregation of γ-cyclodextrin/

C_{60}, so the fully aggregated fullerene preparation exhibited no toxicity toward lens cells.

The polyhydroxylated fullerenes were efficiently internalized by human lens and retinal pigment epithelial cells (Taroni et al. 2011). At lower concentrations, fullerol induced oxidative damage to human lens epithelial cells upon photoirradiation with either UVA or visible light, while higher doses were cytotoxic even in the dark, although to a lesser degree (Roberts et al. 2008). Irrespective of the presence of light, the mechanism of cell death was oxidative stress–mediated apoptosis. Similarly, fullerol was toxic to retinal pigment epithelial cells both in the dark and after exposure to visible light at high and low concentrations, respectively (Wielgus et al. 2010). The observed toxicity was oxidative stress–mediated and involved both apoptosis and necrosis of retinal cells (Wielgus et al. 2010). These data indicate that the exposure to fullerols, particularly in the presence of sunlight, may lead to lens and/or retinal damage.

In vitro skin toxicity of C_{60}

The C_{60} nanoparticles prepared by solvent exchange with THF, as well as polyvinyl pyrrolidone/C_{60} complex, are readily internalized by human keratinocytes, the latter mainly accumulating in the cytoplasm surrounding the nucleus (Scrivens and Tour 1994; Xiao et al. 2010). The THF/nanoC_{60} significantly reduced the growth of human epithelial keratinocytes, and the observed effect was only slightly increased by exposure to light (Bullard Dillard et al. 1996). Further studies confirmed the toxic activity of THF/nanoC_{60} toward human keratinocytes (Markovic et al. 2007; Zhao et al. 2008a). The application of THF/nanoC_{60} was also toxic to human dermal fibroblasts (Sayes et al. 2004; Markovic et al. 2007). In accordance with the proposed role of THF in the cytotoxicity of THF/nanoC_{60}, the C_{60} suspensions prepared mechanochemically with sodium dodecyl sulfate, γ-cyclodextrin, or polyvinyl pyrrolidone demonstrated no dermal cytotoxicity in vitro (Markovic et al. 2007). Moreover, they were shown to decrease UV irradiation–induced ROS generation, apoptotic DNA cleavage, and melanin production in human epidermal keratinocytes and melanocytes (Xiao et al. 2005, 2007, 2010), making them suitable for the formulation of skin-care products (Lens 2011). However, γ-cyclodextrin/C_{60} was found to exhibit singlet oxygen–mediated oxidative stress in keratinocytes upon UVA irradiation (Zhao et al. 2008a).

The phototoxicity of $C_{60}(OH)_{24}$ was about 60 times lower than that of γ-cyclodextrin/C_{60}, which correlated with the superoxide and singlet oxygen generation by the former and latter, respectively (Zhao et al. 2008b). A more hydroxylated derivative, $C_{60}(OH)_{32}$, in contrast to $C_{60}(OH)_{20}$ and $C_{60}(OH)_{24}$, killed human keratinocytes in the absence of light (Saathoff et al. 2011). In other studies, however, the polyhydroxylated derivatives suppressed UV-induced oxidative stress and DNA damage in human keratinocytes, with the observed cytoprotective activity rising in proportion with the increase in the number of hydroxyl groups (Xiao et al. 2005; Saitoh et al. 2011). It therefore appears that both "dark" cytotoxicity and antioxidant cytoprotection rise with the increase in the functionalization of the fullerene core, the overall effect possibly depending on concentration. The ambient light toxicity of a C_{60} phenylalanine derivative toward human epidermal keratinocytes was accompanied by an inflammatory response, as demonstrated by the induction of several proinflammatory cytokines, such as interleukin (IL)-1, IL-6, and IL-8 (Rouse et al. 2006).

In vitro neurotoxicity of C_{60}

In the absence of overt photoexcitation, various preparations of polymer-coated fullerenes did not display any toxicity toward neuronal cell lines and even promoted neuronal growth factor–induced neurite outgrowth or attenuated the increase in intraneuronal superoxide induced by angiotensin II (Tsumoto et al. 2010; Tong et al. 2011). While hydroxylated and carboxylated fullerenes were also not toxic to neuronal cell lines, they significantly reduced the viability of primary mouse neurons (Ehrich et al. 2011), which suggests that the use of transformed neurons may not be valid for the assessment of fullerene neurotoxicity. However, the subtoxic doses of carboxylated C_{60} protected primary neurons from oxidative stress–mediated death induced by various neurotoxins, thus lending support for the potential use of fullerenes as neuroprotective agents (Dugan et al. 2001). It should be also noted that carboxyfullerenes at non-toxic concentrations inhibited neuronal synthesis of the important neuromodulator nitric oxide, thus demonstrating a potential to alter neuronal function (Wolff et al. 2000).

To conclude this part of the review, it appears that both pristine C_{60} and fullerene derivatives are able to exhibit significant in vitro cytotoxicity toward various cell types, which can be further augmented by exposure to light (Fig. 1). Interestingly, unlike strictly ROS-mediated cytotoxicity of photoexcited fullerenes, their "dark" toxicity might also involve oxidative stress–independent mechanisms, including cell membrane damage, cytoskeleton reorganization, and autophagic digestion of cellular constituents. The concept that pristine C_{60} is more prone to ROS generation and thus more cytotoxic than its derivatized counterparts has been challenged due to the contribution of organic solvent (tetrahydrofuran) residually present in the solvent exchange–prepared "pristine"

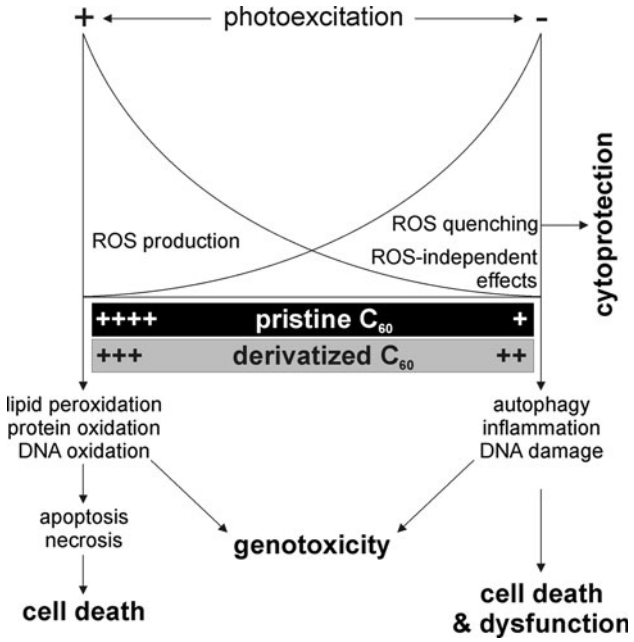

Fig. 1 A simplified overview of the fullerene toxic effects and underlying mechanisms

fullerenes. Nevertheless, the above dichotomy might still apply to photoexcited fullerenes, as some findings indeed demonstrate higher cytotoxicity of pristine C_{60}, possibly as a consequence of better ROS-generating ability and/or production of biologically more reactive ROS (e.g., singlet oxygen instead of superoxide). On the other hand, it appears that the ROS-unrelated "dark" cytotoxicity of functionalized fullerenes might be higher than that of surfactant-/polymer-coated pure C_{60}, presumably due to smaller size and better penetration of cell membranes. It should be noted, however, that suspensions of fullerene derivatives are usually produced and used at much higher concentrations than those containing pristine C_{60}, so further studies are required for the valid comparison of their cytotoxic potencies. Although derivatization of fullerenes is expected to reduce their toxicity and increase cytoprotective efficiency due to generally lower generation and better quenching of ROS, the exceptions have been observed. The type of functional addends could also significantly contribute to the biological activity and toxicity of fullerene derivatives, at least partly by determining their surface charge and aggregation properties.

In vivo toxicity of pristine and derivatized C_{60}

As the site of entry is crucial for the ability of nanoparticles to gain access to systemic circulation and exert local and systemic toxicity, we have analyzed in vivo toxicity of fullerenes according to the exposure route. The in vivo toxicity to C_{60} and its derivatives has been assessed in a relatively limited number of studies after inhalation/intratracheal instillation or parenteral (intraperitoneal and intravenous), oral, dermal, and intraocular administration. Both the toxicity at the site of application and that resulting from the distribution of C_{60} in the organism were investigated, and the data are summarized in Table 2.

Pulmonary exposure

The air samples from urban atmospheres have been shown to contain fullerenes, probably originating from the combustion of coal in power plants (Utsunomiya et al. 2002). The exposure can occur via inhalation at the workplace and from ambient air, but its significance is still uncertain. The main mechanism for the deposition of inhaled nanoparticles in the respiratory tract is diffusion dependent on the particle size (Oberdörster et al. 2005). Several studies assessed the deposition of fullerene nanoparticles in the lungs and internal organs, pulmonary histopathologic features, cellular and biochemical constituents of the bronchoalveolar fluid, and gene expression in the lung tissue after pulmonary exposure to pristine C_{60} and its derivatives.

After intratracheal instillation of pure sonicated C_{60} to mice, the fullerene aggregates were seen by light microscopy in the alveolar and capillary lumen, as well as in pulmonary lymph nodes shortly after exposure, while particles in the alveolar capillaries were observed 6 h after fullerene administration (Naota et al. 2009). Electron microscopy confirmed that fullerene particles were present at the air–blood barrier as early as 5 min after instillation, suggesting that the diffusion process may play a major role in fullerene translocation. The increase in the number of caveolae-like vesicles was evident in both alveolar epithelial and endothelial cells, implicating pinocytosis as the additional translocation pathway. While no signs of lung inflammation, hemorrhage, or histopathologic abnormalities were observed, these findings suggest that inhaled fullerenes may rapidly enter the systemic blood circulation, spreading to other organs and tissues.

After 4 weeks of whole-body inhalation of surfactant (Tween 80)-dissolved pristine C_{60} dispersed in aerosol, C_{60} particles have been found in alveolar macrophages, but no histopathologic abnormalities were observed in the lung tissue of rats (Fujita et al. 2009). Moreover, in comparison with nickel oxide particles, fullerenes only marginally upregulated the expression of genes involved in inflammation, oxidative stress, and apoptosis, implying the absence of severe pulmonary toxicity. In the same experimental setting, only a small transient inflammatory lung infiltration, but no increase in total cell and neutrophil count in bronchoalveolar fluid was observed in rats upon

Table 2 In vivo toxicity of C_{60}

Experimental model	Fullerene type and dose	Main findings	References
Intratracheal instillation (rat)	C_{60} (Tween 80-dispersed) 0.33–3.3 mg/kg, 4 weeks	Inflammatory reaction in the lungs with neutrophils and some eosinophils, ↑neutrophil counts in the BAL fluid, ↑lung expression of genes for neutrophil chemoattractants at 3.3 mg/kg	Ogami et al. (2011) Morimoto et al. (2010b)
Intratracheal instillation (rat)	THF/nanoC_{60} 0.2–3 mg/kg, Single dose	↑Neutrophil counts and lipid peroxidation in the BAL fluid at 1.5 and 3 mg/kg	Sayes et al. (2007)
Intratracheal instillation (rat)	$C_{60}(OH)_{22–24}$ 5–50 mg/kg, 3 days	Bronchitis/alveolitis, ↑neutrophil counts, cellular damage markers in the BAL fluid at 25, 50 mg/kg	Xu et al. (2009a)
Intratracheal instillation (mouse)	C_{60} (solvent exchange in toluene) 0.5–2 mg/kg, single dose	↑Apoptosis in BAL fluid cells, ↑$CD4^+$ T cells in the blood and spleen, ↑TNF, IL-6, IL-12, IFN-γ in the BAL fluid and blood	Park et al. (2010)
Intratracheal instillation (mouse)	$C_{60}(OH)_{20\pm2}$ 0.001–10 mg/kg, single dose	Neutrophil inflammatory response, ↑MCP-1 in the BAL fluid at 10 mg/kg	Roursgaard et al. (2008)
Intratracheal instillation (apolipoprotein E–deficient mice)	C_{60} (sonicated in saline) 2.5 mg/kg, single dose	↑MCP-1, MIP-2 and IL-6 gene expression in the lung tissue	Jacobsen et al. (2009)
Inhalation—whole-body exposure (rat)	C_{60} (Tween 80-dispersed) 0.12 mg/m^3, 4 weeks	↑Expression of genes involved in inflammation, oxidative stress and apoptosis in the lungs	Fujita et al. (2009)
Inhalation—nose only exposure (rat)	C_{60} (milled) NP 2.22 mg/m^3, 10 days MP 2.35 mg/m^3, 10 days	↑BAL fluid proteins (NP group) ↑TNF and IL-1 in the BAL fluid (MP group)	Baker et al. (2008)
Intraperitoneal injection (mouse)	THF/nanoC_{60} 1 mg/kg, single dose	↓Spleen lymphocyte proliferative capacity ↑Melanoma growth	Zogovic et al. (2009)
Intraperitoneal injection (mouse)	$C_{60}O_5(OH)_{18}$ 10–1,000 mg/kg, single dose	LD_{50} = 1200 mg/kg, weight loss, ↓cytochrome P-450-dependent monooxygenase activity in the liver	Ueng et al. (1997)
Intraperitoneal injection (rat)	Polyalkylsulfonated C_{60} 500–1,000 mg/kg (acute) 0.6–60 mg/kg (subacute) Single dose	LD_{50} = 600 mg/kg, diffuse necrosis of tubular epithelium and phagolysosome nephropathy (acute study); phagolysosomal nephropathy (subacute study)	Chen et al. (1998)
Intravenous injection (rat)	Polyalkylsulfonated C_{60} 10, 100 mg/kg; single dose	Phagolysosomal nephropathy at 100 mg/kg	Chen et al. (1998)
Intravenous injection (rat)	MSAD-C_{60} 25 mg/kg, single dose	Death shortly after administration	Rajagopalan et al. (1996)
Intragastric application (rat)	C_{60} (sonicated in saline or corn oil) 0.064 and 0.64 mg/kg Single dose	Oxidatively generated DNA lesions in the liver and lungs at 0.64 mg/kg	Folkmann et al. (2009)

Only the studies demonstrating the adverse effects of C_{60} are presented (for the reports on the absence of toxicity, refer to manuscript text)

BAL bronchoalveolar lavage fluid, *IFN* interferon, *IL* interleukin, *MCP-1* monocyte chemoattractant protein-1, *MIP-2* macrophage inflammatory protein-2, *MP* microparticles, *MSAD-C_{60}* bis(monosuccinimide) derivative of p,p′-bis(2-aminoethyl)-diphenyl C_{60}, *NP* nanoparticles, *THF* tetrahydrofuran, *TNF* tumor necrosis factor

intratracheal inhalation of Tween-dispersed C_{60} (Morimoto et al. 2010b; Ogami et al. 2011). The intratracheal administration of C_{60} at the high dose (>3 mg/kg) caused a significant increase in inflammation areas containing neutrophils and some eosinophils up to 1 week post-application, as well as a transient significant increase in

neutrophils in bronchoalveolar fluid (Morimoto et al. 2010b; Ogami et al. 2011). Therefore, the lung inflammation due to exposure to C_{60} was slight and transient, with no significant pathological changes observed in either lungs or other organs, such as liver, spleen, kidney, brain, nasal cavity, and testis. This is consistent with the distribution pattern of C_{60} nanoparticles after intratracheal instillation/inhalation, showing a transient presence in lung alveolar macrophages and no accumulation in other organs (Shinohara et al. 2010). Thus, the main clearance pathway appears to be phagocytosis by the alveolar macrophages followed by tracheobronchial elimination, but some particles were also seen in the lung epithelial cells, suggesting the second clearance pathway through the lymphatic system.

The intratracheal instillation of the solvent exchange–prepared THF/nanoC_{60} to rats caused no increase in lactate dehydrogenase, protein values, and alkaline phosphatase in bronchoalveolar fluid up to 3 months post-exposure (Sayes et al. 2007). The histopathologic examination revealed normal lung architecture, and an initial increase in bronchoalveolar neutrophil counts at 24 h was not sustained at further time points. Although a significant increase in lipid peroxidation was observed in the bronchoalveolar fluid of rats exposed to THF/nanoC_{60}, the glutathione concentration was not altered, indicating no significant adverse effects. Accordingly, the nasal exposure of mice to Tween/C_{60} aerosol did not influence the half-life of intravenously administrated nitroxide radical, suggesting that pulmonary antioxidant defense was not impaired (Yokoyama et al. 2009).

The exposure to C_{60} aerosol over 10 consecutive days in either nanoparticle or microparticle form did not result in significant changes in the body or organ weight, and histopathologic examination of lungs and other organs did not reveal any microscopic lesions in both groups (Baker et al. 2008). Although some alveolar macrophages contained intracytoplasmic brown pigment consistent with the presence of C_{60}, the analysis of bronchoalveolar fluid revealed no significant differences in the cell count in comparison with negative control, and no fullerenes were detected in the blood. However, the concentration of proinflammatory cytokines tumor necrosis factor (TNF) and IL-1 were significantly increased in the microparticle group, indicating the presence of an inflammatory response. Accordingly, intratracheal instillation of solvent exchange (toluene/H_2O)-prepared C_{60} suspension to mice caused immune activation, reflected in the increase in expression of MHC class I and II molecules in lungs and $CD4^+$ T-cell counts in blood and spleen, as well as in the secretion of proinflammatory cytokines (TNF, IL-1, IL-6) and Th1 cytokines IL-12 and interferon (IFN)-γ in the bronchoalveolar fluid and blood (Park et al. 2010). With the exception of IFN-γ, which persisted at high levels in both lungs and blood, the concentrations of other cytokines rapidly declined following the initial increase. The observed immune activation was associated with lung toxicity, as demonstrated by the cell cycle disturbances (G_1 block), increase in number of apoptotic cells in the bronchoalveolar fluid, and the elevated expression of genes related to tissue damage, such as matrix metalloproteinase. The ability of intratracheally instilled C_{60} to cause lung inflammation was confirmed by using water-sonicated C_{60} in apolipoprotein E knockout mice, which are more susceptible to toxic damage, presumably due to a higher permeability of their alveolar–blood barrier or inflammatory target cells to nanoparticles (Jacobsen et al. 2009). The gene expression of monocyte chemoattractant protein-1, involved in monocyte chemotaxis and maturation to macrophages, as well as that of macrophage inflammatory protein-2, which recruits neutrophils, was significantly higher in the lung tissue 24 h after administration. This was associated with the increased expression of the proinflammatory cytokine IL-6, but no significant alterations in the bronchoalveolar fluid cellular composition or cellular DNA damage were observed. Of note, the inflammation caused by C_{60} instillation resulted in a substantially weaker inflammatory response in comparison with other carbonaceous particles.

As for the pulmonary exposure to derivatized C_{60}, the intratracheal instillation of fullerol (10 mg/kg) after 24 h elicited a neutrophil-driven inflammatory response associated with the increased production of macrophage inflammatory protein-2 (Roursgaard et al. 2008). The tenfold lower dose of fullerol did not give rise to lung inflammatory response and even attenuated ROS-dependent pulmonary inflammation induced by quartz. Therefore, it appears that fullerol at lower doses may have protective anti-inflammatory action, but at higher doses, it exhibits proinflammatory effects. Similarly, intratracheal administration of high doses (> 25 mg/kg) of fullerol to rats for 3 days caused severe bronchitis and alveolitis, associated with an increase in neutrophil count in the bronchoalveolar fluid and blood (Xu et al. 2009a). The biochemical markers of cellular damage (lactate dehydrogenase, alkaline phosphatase), total protein and albumin concentrations, as well as proinflammatory cytokines (TNF, IL-1 and IL-6) and the indicators of oxidative/nitrosative stress, were also increased in the bronchoalveolar fluid of fullerol-treated rats. On the other hand, rats exposed to the lower dose of fullerol (5 mg/kg) did not display histopathologic changes or inflammatory infiltration in the lung tissue. Accordingly, another study in rats intratracheally instilled with fullerol (up to 3 mg/kg) for 24 h demonstrated no lung tissue damage/lipid peroxidation and only a transient inflammatory response, evidenced by an increase in the neutrophil count (Sayes et al. 2007).

Therefore, it seems that inhalation/instillation of both pristine and functionalized (hydroxylated) C_{60} can induce mild transient lung inflammation in rodents, while sustained inflammation accompanied by tissue damage was only rarely observed at high doses.

Parenteral (intraperitoneal) administration

After a single intraperitoneal injection of detergent (Tween 60)-solubilized C_{60} (500 mg/kg) to rats, liver was the principal site of fullerene accumulation, which was mainly seen in Kupffer cells and rarely in hepatocytes and hepatic stellate cells (Gharbi et al. 2005). The microscopic examination revealed normal parenchymal architecture without inflammation or fibrosis, while the normal levels of serum alanine aminotransferase confirmed the absence of parenchymal cell damage. Moreover, pretreatment with C_{60} aqueous suspension protected the liver from ROS-mediated acute toxicity of the profibrotic chemical CCl_4 (Gharbi et al. 2005). The THF/nanoC_{60} labeled with ^{125}I was found to readily accumulate in liver, spleen, heart, lung, kidney, intestine, muscle, and bone tissues after intraperitoneal injection to rats (Zogovic et al. 2009). The daily intraperitoneal dose of 0.25 μg/kg nanoC_{60} was found to be non-toxic, as evaluated by normal weight gain and the absence of symptoms over the 2 weeks of application. Surprisingly, in contrast to in vitro cytotoxicity of THF/nanoC_{60} toward melanoma cells, its intraperitoneal administration significantly enhanced melanoma growth. The tumor-promoting effect was presumably due to the interference of C_{60} with the host antitumor immune defense, as indicated by the increased production of nitric oxide and its possible role in reducing the splenocyte proliferative responses.

Regarding the parenteral toxicity of C_{60} derivatives, the water-soluble polyalkylsulfonated C_{60} administered to rats via single intraperitoneal injection was found to have an LD_{50} of 600 mg/kg (Chen et al. 1998). Microscopic examination of kidneys revealed a diffuse necrosis of tubular epithelium in the outer cortex, presumably due to phagolysosome overload nephropathy. Although the kidney was a primary organ of fullerene rapid elimination and consequent toxicity, the numerous pigment-laden macrophages were also seen in liver, spleen, and thymus. In another study, however, the treatment of rats with polyalkylsulfonated C_{60} protected proximal tubule cells from ischemia-induced oxidative damage (Chien et al. 2001). After a single intraperitoneal injection (500 mg/kg) of the fullerene derivative synthesized by using dipolar trimethylenemethane, which undergoes cycloaddition to C_{60}, all mice survived for 1 week, although symptoms of discomfort, including writhing with stretching of the trunk, as well as some weight loss occurred (Yamago et al. 1995). The LD_{50} value of polyhydroxylated C_{60} administered to mice in a single intraperitoneal injection was estimated at 1,200 mg/kg (Ueng et al. 1997). Fullerol reduced the activity of cytochrome P450–dependent liver monooxygenases, possibly by inhibiting the catalytic activity of the enzyme, although the increase in enzyme destruction or suppression of its de novo synthesis could not be excluded. At lower doses (100 mg/kg), intraperitoneal fullerol did not influence the peripheral blood cell count, hemoglobin, and hematocrit levels in rats, but it decreased antioxidative capacity of erythrocytes in oxidative stress induced by doxorubicin administration (Milic et al. 2009). A further reduction in the fullerol dose ($\leq$ 50 mg/kg) led to a complete loss of any toxicity and enabled protection against oxidative stress–mediated cardiotoxicity, hepatotoxicity, and nephrotoxicity in doxorubicin-treated rats (Injac et al. 2008, 2009).

Parenteral (intravenous) administration

The intravenous administration of fullerenes has mainly been used to assess their biodistribution and/or anticancer action. ^{125}I-labeled THF/nanoC_{60} injected intravenously to rats was promptly delivered throughout the body, with the highest uptake recorded in the liver and spleen (Nikolic et al. 2009). The aqueous suspension of C_{60} fullerene with ^{14}C-labeled core was rapidly cleared from circulation, with over 90 % of the initial activity accumulated in the liver and the rest distributed in the spleen, lung, and muscle, without entering the brain (Bullard Dillard et al. 1996). Following an intravenous injection to mice, ^{125}I-labeled C_{60}/polyethylene glycol conjugate exhibited higher accumulation and the prolonged retention in subcutaneously implanted fibrosarcoma than in normal skin and muscle, causing photoinduced tumor necrosis without affecting the overlying skin (Tabata et al. 1997). In a study investigating the role of C_{60} in atherosclerosis progression, mechanochemically prepared C_{60}/polyethylene glycol readily accumulated in the atherosclerotic plaque upon intravenous injection, causing further photoinduced atherosclerotic lesions in arteries with previous intimal injury (Nitta et al. 2008).

Similar to the biodistribution data of intravenously injected pristine C_{60} preparations, ^{125}I-labeled fullerol was rapidly cleared from circulation to accumulate mainly in kidney, liver, spleen, and bones, followed by elimination in urine and feces (Ji et al. 2006). In mice implanted with various human tumors, the uptake of fullerol in the tumor was higher than in normal tissues (Ji et al. 2006). The ^{14}C-labeled fullerene derivative synthesized by cycloaddition of trimethylenemethane to C_{60} was rapidly cleared from blood, accumulating preferentially in liver and much less in spleen and kidney, being mainly excreted in feces (Yamago

et al. 1995). The clearance of ^{14}C-labeled C_{60} ammonium salt from circulation was also rapid, but somewhat slower than that of pristine C_{60} (Bullard Dillard et al. 1996). The highest accumulation was measured in liver, followed by lung, muscle, and skin, while this fullerene derivative, like its parent C_{60} compound, did not cross the brain–blood barrier (Bullard Dillard et al. 1996). The bis(monosuccinimide) derivative of p,p′-bis(2-aminoethyl)-diphenyl C_{60}, administered intravenously to rats, rapidly bound to plasma proteins and extensively accumulated in various tissues in the absence of significant renal clearance (Rajagopalan et al. 1996). The high dose of this C_{60} preparation (25 mg/kg) resulted in the death of two rats 5 min after intravenous application, but the possibility that the toxicity was due to an interaction between C_{60} and the vehicle (dimethylsulfoxide) could not be ruled out. A single intravenous injection of water-soluble polyalkylsulfonated C_{60} (100 mg/kg), similar to the results obtained with intraperitoneal injection, caused moderate phagolysosomal nephropathy in rats (Chen et al. 1998).

The above results demonstrate that upon parenteral administration, both pristine and functionalized fullerenes are rapidly cleared from circulation, accumulating preferentially in the liver, spleen, and kidneys, followed by the elimination in urine and feces. Accordingly, the toxic effects are usually observed in these organs, including inhibition of liver cytochromes, splenic immunosuppression, and phagolysosomal nephropathy. No substantial amount of C_{60} was apparently able to cross the blood–brain barrier and cause neurotoxicity. The toxic effects were usually observed only at high concentrations (>500 mg/kg), although some C_{60} derivatives were toxic at 10- to 20-fold lower doses. While there was no evidence that the toxicity was dependent on oxidative stress, non-toxic concentrations of both pristine and derivatized C_{60} exerted antioxidant tissue-protective effects. Importantly, pristine C_{60} was efficient in selective photodynamic killing of tumor cells in vivo, but in the absence of photoactivation, the immunomodulatory events leading to promotion of cancer growth were observed.

Oral administration

Only few studies thus far investigated the absorption, distribution, excretion, and toxicity of orally administered C_{60} in rodents. A fairly high dose (2,000 mg/kg) of detergent (Tween 80)-solubilized C_{60} did not cause any signs of toxicity after single oral administration to rats, and no tissue abnormalities were seen at necropsy (Mori et al. 2006). On the other hand, much lower dose (0.64 mg/kg) of pure C_{60} administered orally in saline or corn oil significantly increased the amount of oxidatively damaged DNA in the liver and lungs (Folkmann et al. 2009). No alterations of the DNA repair activity were observed, indicating that the level of DNA damage was not underestimated as a consequence of increased repair. This finding suggests that fullerene particles can be absorbed from the gastrointestinal tract to blood circulation and secondary organs, although no direct evidence was found to support this assumption. A single oral dose (up to 2,500 mg/kg) of polyalkylsulfonated C_{60} did not cause any mortality in rats (Chen et al. 1998). While orally administered ^{14}C-labeled C_{60} derivative was almost completely excreted in the feces within 48 h, a trace amount was partially metabolized and excreted into the urine, indicating that translocation through the gastrointestinal wall still occurred, albeit to a small degree (Yamago et al. 1995). It therefore appears that gastrointestinal absorption, and consequently systemic toxicity, of orally ingested C_{60} are fairly low. However, the unwanted systemic effects, such as pristine C_{60}–mediated oxidative DNA damage, are still possible and deserve special attention.

Dermal exposure

The substantial antioxidant properties make fullerenes potentially suitable for cosmetic applications. Molecular complexation of fullerenes with cyclodextrins, polyvinyl pyrrolidone, and liposomes is of particular interest for the preparation of cosmetic preparations like rejuvenating, whitening/lightening, and sun protection skin products. Thus, the skin is considered the main exposure route for consumers. The study performed on pigs demonstrated that the skin penetration of C_{60} depends on the vehicle used, with higher penetration observed for chloroform than toluene or cyclohexane preparations, and no penetration found after C_{60} application in mineral oil (Xia et al. 2010a, b). Therefore, the vehicle selection might be of pivotal importance for the safety of topically applied pristine C_{60}. On the other hand, the organic solvent–free liposome-incorporated fullerene was found to penetrate the epidermis without entering the dermis of human biopsy samples, implying no necessity for considering systemic toxicity of this compound (Kato et al. 2009b; 2010). The patch testing to assess the skin irritant potential of fullerene soot in 30 volunteers who reported irritation and allergic susceptibilities demonstrated no skin irritation after a 96-h exposure (Huczko et al. 1999). Similarly, the administration of C_{60} in propylene glycol did not induce skin irritation in rabbits or guinea pigs irrespectively of exposure to UV light, and no skin reactions were observed in the patch test in human subjects (Aoshima et al. 2009). Moreover, the C_{60} dispersed in polyvinyl pyrrolidone was not toxic to UV-B-irradiated mouse skin and even reduced oxidative stress–mediated apoptotic skin damage induced by UV irradiation (Ito et al. 2010). These results indicate no toxicity and

possible antioxidant protective action of pristine C_{60} in the skin. As for the skin toxicity of derivatized C_{60}, with the exception of the report showing that amino acid-functionalized fullerenes were able to penetrate into the dermis of mechanically flexed skin (Rouse et al. 2007), we did not identify any relevant in vivo studies.

Ocular exposure

Although the eye, due to exposure to light, could be particularly sensitive to fullerene phototoxicity, only few studies thus far addressed the ocular effects of C_{60} preparations. In the eye irritation test performed in rabbits, instillation of a fullerene soot suspension for up to 72 h was observed to have no ocular toxicity (Huczko et al. 1999). On the other hand, conjunctival discharge and redness were observed at 1 h after application of C_{60} into the lower conjunctival sac of rabbit's eye, but the symptoms disappeared after 48 h (Aoshima et al. 2009). The irritation observed was probably caused by the administration of insoluble powder rather than the toxic effect of fullerene. Based on these studies, pristine C_{60} does not appear to have significant ocular toxicity. To the best of our knowledge, similar studies have not been performed with fullerene derivatives.

In vitro and in vivo genotoxicity of pristine and derivatized C_{60}

Finally, we evaluate the data on the genotoxicity of fullerenes, which could occur without overt acute or chronic toxicity, but could have important implications for human health in terms of carcinogenesis and hereditary disorders. The most relevant property of fullerenes with regard to their genotoxic potential is their ability to generate ROS. Two principle modes for particles' genotoxic action have been introduced, referred to as primary and secondary genotoxicity (Schins and Knaapen 2007). Primary genotoxicity is defined as genetic damage elicited by particles in the absence of inflammation, deriving either from intrinsic particle-associated ROS or from ROS generated through the interaction of particles with the target cells. The secondary genotoxicity can be defined as genetic damage resulting from ROS and reactive nitrogen species (RNS) generated consequently to particle-elicited inflammation. The data presented in the present review, showing the ability of fullerenes to cause oxidative stress–mediated cell damage, as well as inflammatory response, indicate their potential to mediate both primary and secondary genotoxicity. A number of studies have assessed the genotoxic effects of C_{60}, mainly by using the reverse mutation test in bacteria (Ames test), single cell gel electrophoresis (Comet assay), and the micronucleus test or measuring the oxidative DNA damage and mutation frequency in mammalian cells in vitro and in vivo.

Although solvent exchange–prepared THF/nanoC_{60} was found to yield positive responses in bacterial genotoxicity tests, the incubation with human hepatocarcinoma cells did not result in disruption of nucleic acid structure and covalent DNA adducts formation (Matsuda et al. 2011). In another study, Comet assay demonstrated lymphocyte genotoxicity of water-stirred and solvent exchange–prepared (ethanol/H_20) C_{60} suspensions (Dhawan et al. 2006). Furthermore, water-stirred C_{60} was more potent that its solvent exchange counterpart, thus eliminating any possibility of solvent contribution to fullerene genotoxic effect, at least in this case. The mutagenic potential of C_{60} water suspension in mouse embryonic fibroblasts was apparently dependent on endocytosis, followed by nitric oxide synthase–dependent induction of cyclooxygenase-2 (Xu et al. 2009b). Although sonicated C_{60} failed to induce DNA strand breaks in mouse lung epithelial cells, a significant increase in oxidation of purines was observed (Jacobsen et al. 2008). The treatment for human lung carcinoma cells with detergent (Tween 80)-solubilized C_{60} caused an increase in the micronuclei number, consistent with the Comet assay–confirmed DNA damage and increase in mutation frequencies in mice lungs after intratracheal instillation (Totsuka et al. 2009). Accordingly, the genotoxicity of intratracheally instilled saline-sonicated C_{60} was demonstrated by the Comet assay in bronchoalveolar fluid cells from apolipoprotein E knockout mice (Jacobsen et al. 2009). The in vivo genotoxic potential of pristine fullerenes was confirmed by the ability of C_{60} sonicated in saline or corn oil to induce oxidative DNA damage in the liver and lungs following intragastric administration (Folkmann et al. 2009). In contrast to the above reports, several studies found no evidence for the in vitro or in vivo genotoxicity of pure fullerenes. Namely, the mechanochemically prepared polyvinyl pyrrolidone/C_{60} complex did not induce chromosomal aberrations in cultured hamster lung cells and was non-mutagenic in the Ames test unless photoexcited (Sera et al. 1996; Aoshima et al. 2010). Similarly, C_{60} dispersed in carboxymethylcellulose sodium failed to induce significant chromosomal aberrations in hamster lung cells or reverse mutations in bacteria, regardless of light irradiation (Mori et al. 2006; Shinohara et al. 2009). Accordingly, Tween 80-solubilized C_{60} displayed no in vivo genotoxicity in the lungs or bone marrow of mice after intratracheal or intragastric instillation, respectively (Shinohara et al. 2009; Ema et al. 2012).

As for the genotoxicity of derivatized C_{60}, the chronic treatment (80 days) with low doses (up to 1,000 pg/ml) of fullerol $C_{60}(OH)_{24}$ caused genotoxic effects in germinative and somatic mammalian cells, as evidenced by the increase

in the number of micronuclei (Niwa and Iwai 2006). It was proposed that the unsuccessful chromosome division, rather than direct chromosome lesion by genotoxic ROS, was responsible for the observed effect. Conversely, $C_{60}(OH)_{24}$ was found to decrease the number of micronuclei and chromosomal aberrations in both undamaged and mitomycin C–treated hamster ovary cells, even when applied at micromolar concentrations (Mrdanovic et al. 2009). While C_{60} carboxylic acid amine salt was found to induce DNA cleavage at guanine bases upon photoirradiation (Tokuyama et al. 1993), the mutagenic potential of other fullerene derivatives (carbethoxy-methanofullerene, carbmethoxy-methanofullerene, and fulleropyrrolidine) was found to be insignificant in Ames test (Babynin et al. 2002).

The aforementioned studies demonstrate conflicting results regarding C_{60} genotoxicity. While the choice of the experimental model and methods for the preparation of fullerene suspensions and/or assessing genotoxicity is the logical source for the most of the observed discrepancies, this could not apply to Tween 80-solubilized C_{60}, for which the conflicting findings have been reported in the same experimental model (intratracheal instillation) and using the same methodology (Comet assay) (Totsuka et al. 2009; Ema et al. 2012). It is therefore important to identify which minor differences in apparently similar experimental systems might be responsible for the opposite results regarding fullerene genotoxicity. A number of issues critical for the genotoxicity testing have to be considered, including appropriate characterization of C_{60} solutions, dose–response relationships, and correlation of the in vitro findings with those observed in vivo (Greim and Norppa 2010). Interestingly, there are indications that some genotoxic effects of fullerenes might be ROS-independent, so further studies on genotoxicity of fullerenes and underlying mechanisms are clearly required.

Concluding remarks

The above-presented results indicate that, in addition to the protective antioxidant effects, both pristine and functionalized fullerenes display a range of activities that can cause cell death or dysfunction (summarized in Fig. 1). Due to a relatively limited number of studies performed with each of the fullerene preparations, it is presently unrealistic to make definite conclusions about their toxicological behavior. However, it appears that most of the pristine and functionalized fullerene preparations are not overtly toxic unless photoexcited or used at very high concentrations that are unlikely to be encountered environmentally or during therapy. While THF/nanoC_{60} seems to be an exception, its toxic activity apparently depends on the residual solvent, thus emphasizing the issue of the purity of fullerene preparations. The lack of studies that systematically compare the toxicity of pristine and derivatized C_{60} in the same experimental system makes difficult to judge the impact of the fullerene core functionalization on its toxic properties. Nevertheless, both pure C_{60} and functionalized C_{60} exert oxidative stress–mediated photodynamic cytotoxicity, while some C_{60} preparations are able to kill cells even in the absence of light. The latter is usually associated with ROS-independent cell membrane damage and/or induction of autophagy, the latter apparently being a common denominator of cellular response to nanoparticle uptake (Zabirnyk et al. 2007). It seems safe to conclude from the above results that the differences in cytotoxic potency and underlying mechanisms displayed by various fullerene preparations are mainly due to some physicochemical characteristics, such as particle size (surface/volume ratio), surface charge, and aggregation properties, which are partly, but not exclusively, related to the "pure/functionalized" dichotomy. Interestingly, another discrepancy, between the fullerene toxicity observed in vitro and in vivo, readily emerges from the available data. Namely, it appears that fullerene preparations that exert in vitro toxicity, including the extremely toxic THF/nC_{60}, are substantially less or not toxic in vivo. The underlying reasons might include the presence of various physiological barriers, binding to and inactivation by serum proteins, as well as the absence of light required for ROS generation and subsequent toxicity. Even the light-exposed and therefore presumably more vulnerable sites such as skin or eye, were apparently not significantly damaged in contact with fullerenes, although additional studies involving direct exposure to sunlight are needed. However, as fullerenes, depending on the exposure route, have been found to extensively accumulate in lungs, liver, spleen, and kidneys, a special care has to be taken as to their possible toxic effects in these organs. Moreover, both pure and functionalized C_{60} displayed ROS-dependent and ROS-independent genotoxicity in vitro, and the low doses of pristine C_{60}, although causing no obvious acute or chronic macrotoxicity, were found to induce oxidative DNA damage in the internal organs. These findings warrant caution regarding the possibility that chronic low exposure to fullerene nanoparticles might increase risk of cancer, hereditary disorders, and organ dysfunction in the absence of overt toxicity. However, the data on the genotoxicity of fullerenes are conflicting, so additional studies are clearly required. The potential risks of the chronic fullerene exposure are further emphasized by numerous reports confirming the ability of both pristine and derivatized C_{60} to induce inflammatory responses in vivo. Since chronic inflammation and ensuing oxidative stress have been implicated in cancerogenesis, autoimmunity, and metabolic

and cardiovascular disorders (Bottasso et al. 2009), the effects of low-grade inflammation or immunomodulation associated with chronic fullerene exposure need to be explored. The interaction of fullerene nanoparticles with the immune system probably occurs via uptake by immune cells such as macrophages and dendritic cells, which triggers intracellular signaling leading to the production of inflammatory mediators (Marano et al. 2011). Therefore, the strategies for the development of fullerenes as therapeutic agents should include complexation with appropriate molecules and other approaches aimed at reducing their recognition and internalization by the immune cells. Finally, the assessment of the consequences of fullerene interaction with mammalian cells and tissues is further complicated by the possibility that some pathological conditions might reduce the threshold for or enable the occurrence of fullerenes' unwanted effects. In the future, we will need more mechanistically driven, structure–activity relationship-based studies to be able to estimate more closely the possible risks that environmental or therapeutic fullerene exposure might pose to human health.

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