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Review

Biomedical potential of the reactive oxygen species generation and quenching by fullerenes (C_{60})

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ABSTRACT

Fullerene (C_{60}), a third carbon allotrope, is a classical engineered material with the potential application in biomedicine. One of the biologically most relevant features of C_{60} is the ability to quench various free radicals, behaving as a "free radical sponge". Conversely, photosensitization of C_{60} leads to its transition to a long-lived triplet excited state and the subsequent energy or electron transfer to molecular oxygen, yielding highly reactive singlet oxygen ($^1O_2$) or superoxide anion ($O_2^{\bullet-}$), respectively. These reactive oxygen species (ROS) react with a wide range of biological targets and are known to be involved in both cellular signaling and cell damage. Therefore, the dual property of fullerenes to either quench or generate cell-damaging ROS could be potentially exploited for their development as cytoprotective or cytotoxic anticancer/antimicrobial agents. However, the attempts to that effect have been hampered by the extremely low water solubility of C_{60}, and by the fact that solubilization procedures profoundly influence the ROS-generating/quenching properties of C_{60}, either through chemical modification or through formation of complex nanoscale particles with different photophysical properties. We here analyze the mechanisms and biological consequences of ROS generation/quenching by C_{60}, focusing on the influence that different physico-chemical alterations exert on its ROS-related biological behavior.

1. Introduction

Fullerenes, the large carbon cage molecules considered to be three-dimensional analogues of benzene, represent a third carbon allotrope [1]. The most abundant form of fullerenes is buckminsterfullerene (C_{60}) with 60 carbon atoms arranged in a spherical structure (Fig. 1). The shape of the molecule, known as truncated icosahedron, resembles that of a soccer ball, containing 12 pentagons and 20 hexagons, in which every carbon atom forms bond to three other adjacent atoms through sp^2 hybridization [1,2]. There are two types of bonds in the fullerene: C_5–C_5 single bonds in the pentagons and C_5–C_6 double bonds in the hexagons (Fig. 1). The unique physical and chemical features of C_{60}, the most representative member of the fullerene family, have recently incited a considerable hope of its possible use in various fields of biomedicine. Many fullerene-based compounds with different biological targets have been synthesized, displaying a range of biological activities potentially useful in anticancer or antimicrobial therapy, cytoprotection, enzyme inhibition, controlled drug delivery and contrast- or radioactivity-based diagnostic imaging (reviewed in Refs. [3,4]).

One of the biologically most relevant features of C_{60} is the ability to function as a "free radical sponge" and quench various free radicals more efficiently than conventional antioxidants [5], a property that was attributed to a delocalized π double bond system of the fullerene cage. On the other hand, illumination of C_{60} with visible or UV light fosters its transition to a long-lived triplet excited state and the subsequent energy transfer to molecular

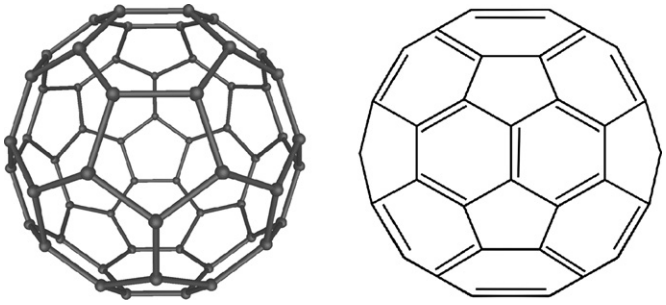

Fig. 1. The structure of C_{60}.

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oxygen, yielding a highly reactive singlet oxygen ($^1O_2$) [6,7]. Singlet oxygen and other reactive oxygen species (ROS) react with a wide range of biological targets and are known to be involved in both cellular signaling and cell damage [8]. This dual property of C_{60} to either quench or generate cell-damaging ROS could be therefore exploited for its development as a cytoprotective or cytotoxic anticancer/antimicrobial agent. On the other hand, the potential ROS-dependent toxicity of C_{60} must be thoroughly explored and controlled, since it can compromise the biocompatibility of fullerene-based drug-delivery or tissue-scaffolding systems, as well as the envisaged widespread use of C_{60} in consumer products. However, the investigation of these issues has been hampered by the extremely low water solubility of C_{60} and the fact that derivatization of the fullerene core with various functional groups and other solubilization procedures can change the photophysical properties and ROS-generating/quenching capacity of C_{60}. Moreover, although fullerene molecule, with the diameter of 0.7 nm, does not fulfill the size criteria for nanoparticles [9], many fullerene preparations in solution form nanoscale aggregates with distinctly different properties from the parental molecule, which further increases the complexity of fullerenes' behavior in biological systems.

In this review, we analyze the basic physical chemistry, as well as biological consequences of C_{60}-mediated ROS generation/quenching, with the focus on the influence that physico-chemical alterations of the fullerene molecule exert on its ROS-related biological behavior.

2. Physical chemistry of C_{60}-mediated ROS generation and quenching

A unique electron configuration of molecular oxygen, characterized by two single-occupied antibonding orbitals that contain electrons with parallel spins, could give rise to three energetically close electronic states – the Σ triplet ground state and the excited Σ and Δ singlet states [10]. The $O_2(^1\Delta_g)$ state, in which both electrons are paired in a single orbital, has lower energy, but considerably longer lifetime than Σ excited state, and it is identified as the metastable O_2 species commonly known as singlet oxygen [11]. Because of the extremely high oxidizing ability resulting from a removal of the spin restriction, singlet oxygen displays significant reactivity in a variety of chemical and biochemical reactions [8].

The most common mean of singlet oxygen generation is photosensitization, a process of energy transfer to triplet ground state oxygen from an excited state of a sensitizer, formed by the absorption of light in a specific wavelength region [12]. Singlet oxygen photosensitization is highly favoured in nature due to very special electronic configuration of molecular O_2, the fact that excitation energies of triplet and singlet oxygen are lower than the energies of triplets of many organic molecules, and the very small size of the O_2 molecule that enables its rapid diffusion in many media. Moreover, because the intramolecular transition of sensitizer from the excited to ground state is spin-forbidden, the excited states generally have long lifetimes, allowing for complete quenching by O_2 in air-saturated solution. A principal measurable property of a photosensitizer is the quantum yield of singlet oxygen, defined as number of $^1O_2$ molecules per one photon of absorbed light. Fullerenes are extremely efficient singlet oxygen generators with the quantum yield of $^1O_2$ that is near unity. Fullerenes absorb strongly in the UV and moderately in the visible regions of the spectrum [2,13]. The singlet excited state of C_{60} ($^1C_{60}^*$), initially formed upon light excitation, undergoes intersystem crossing to triplet state ($^3C_{60}^*$) that can be efficiently quenched by molecular oxygen to generate large amounts of singlet oxygen [6]. However, the excited triplet state of fullerene is an excellent electron acceptor, and the reduced fullerene triplet ($^3C_{60}^{\bullet-}$) can readily transfer an electron to molecular oxygen forming superoxide anion radical $O_2^{\bullet-}$ [14]. This type of

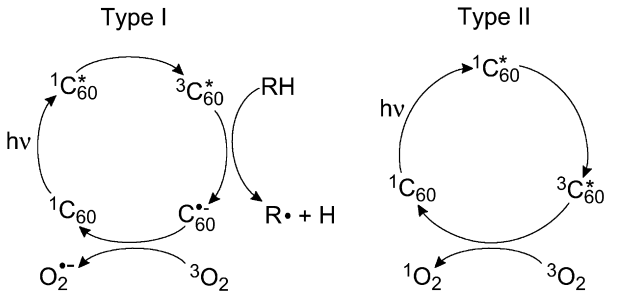

Fig. 2. Schematic representation of Type I (charge transfer) and Type II (energy transfer) photochemical mechanisms (* denotes excited singlet and triplet states of C_{60}).

reaction, in contrast to singlet oxygen generation usually observed in organic solvents (e.g. benzene or toluene), preferentially occurs in polar solvents, particularly in the presence of reducing agents such as NADH [15]. These two pathways, yielding singlet oxygen and superoxide anion, are analogous to the two main photochemical reaction types known as Type II and Type I photochemical mechanisms, respectively (Fig. 2).

In spite of their ability to generate singlet oxygen and superoxide anion when photoexcited, fullerenes are also considered as promising agents for deactivation of various ROS [3]. While many researchers refer to fullerene cage as to a "free radical sponge", this notion does not seem fully substantiated when reactive oxygen intermediates are concerned. Namely, the original work that described the ability of the fullerene core to accept up to 34 free radical moieties actually dealt with carbon-centered (i.e. benzyl and methyl), but not oxygen radicals [5]. Actually, fullerenes have been shown to quench singlet oxygen quite weakly, with an approximate rate constant of only $6.1 \times 10^4\,L\,M^{-1}\,s^{-1}$ [16] or $5 \times 10^5\,L\,M^{-1}\,s^{-1}$ [6], depending on the solvent used. The underlying mechanism was unlikely to involve chemical reaction, because no loss of starting material or formation of new products occurred during photosensitization [6]. It is more likely that the observed effect involved physical quenching due to energy transfer, in which singlet oxygen was deactivated by conversion of its electronic excitation energy into vibration of O_2 and C_{60}. Apart from this weak quenching of singlet oxygen, no study thus far, at least to our knowledge and according to the recent report [17], has demonstrated a significant scavenging capacity of pure, underivatized C_{60} towards major oxygen-centered radicals. It is therefore plausible to consider the hypothesis that the ROS-deactivating capacity of various fullerene preparations might not be entirely related to the fullerene core itself, but also to the attached functional groups or various modifying agents introduced to provide water solubility. In the rest of the paper, we will try to analyze how different solubilization procedures can affect performance of fullerenes as ROS-producing and ROS-quenching agents in various biological settings.

3. Generation and deactivation of ROS by C_{60} in aqueous solutions

Fullerenes are hydrophobic molecules best dissolved in benzenes, naphthalenes and alkanes, so potential biomedical applications of fullerenes have been hampered by their extremely poor solubility in polar solvents. Therefore, four main approaches have been developed for the transfer of fullerenes to water:

(1) Chemical modification of the fullerene carbon cage by attachment of various functional groups (e.g. –OH, NH_2, –COOH), so that fullerene molecule can establish bonds with water via hydrophilic functional adducts [18–21].

(2) Incorporation of fullerenes into water-soluble supramolecular structures using surfactants or other modifying agents, such as calixarenes or cyclodextrins [22–25]. In most cases fullerene core is completely covered by modifying agent and can hardly come in contact with water.
(3) Solvent exchange method that uses volatile water-miscible organic solvents to dissolve fullerene, while in the next step water is added and the solvent evaporated, leaving fullerene aggregates in water suspension [26–28]. Ultrasonic treatment could be used to promote transfer of fullerene from a nonpolar solvent (e.g. toluene) to water [29,30]. The structure of fullerene is not changed and the fullerene core can freely collide with water molecules.
(4) Long-term stirring of pure C_{60} in water [30,31]. While this method enables the investigation of pristine C_{60} in water, its disadvantages include formation of large aggregates and low fullerene concentration.

It should be noted that methods 2–4 mainly result in formation of nanoscale fullerene particles with the sizes ranging from a few tenths to several hundreds of nanometers. This might hold true even for the γ-cyclodextrin-bicapped C_{60}, which theoretically should form a true solution rather than an aggregated suspension. Namely, the atomic force microscopic analysis of γ-cyclodextrin/C_{60} "solution" in water revealed the presence of particles having size up to 50 nm (Markovic et al., unpublished), which is consistent with the reported tendency of γ-cyclodextrin to form aggregates in solution bound together by a network of hydrogen bonds [32]. Although some derivatized fullerenes can also form aggregates in water [33,34], because of clarity we will refer to functionalized fullerene derivatives as to water-soluble fullerenes. On the other hand, the term "fullerene nanoparticles" (or prefix "nano") will be restricted to surfactant-coated, solvent exchange- or long-term stirring-prepared fullerene water suspensions. A schematic representation of the structure of different C_{60} preparations is given in Fig. 3.

3.1. Water-soluble C_{60} derivatives

In recent years there has been much interest in studying potentially bioactive water-soluble fullerene derivatives, with emphasis on their capacity to generate or quench ROS. Various C_{60} derivatives, including polyhydroxylated C_{60} [fullerol or fullerenol – $C_{60}(OH)_n$, $n = 2$–24] and malonic acid derivatives [carboxyfullerene – $C_{60}[C(COOH)_2]_n$, $n = 1$–6], have been found to readily produce $^1O_2$ and/or $O_2^{\cdot-}$ upon photosensitization with UV, plain visible or laser light [35–38]. Other derivatives such as hexasulfobutyl-C_{60} and some fullerene–sugar conjugates have also displayed very efficient photosensitizing capacity, which, in the case of the former, was comparable to that of a clinically approved photosensitizer photofrin [39–41]. Several studies have examined the influence of chemical modification on various photophysical properties of C_{60} and found them markedly affected by interruption of the fullerene core. In a series of malonic acid derivatives, it was found that singlet oxygen yields tend to decrease with increasing number of addends, dropping to the values near 0.1 for hexa-adduct [35]. Accordingly, disaccharide C_{60} derivatives produce less singlet oxygen than fullerene monosaccharides [39]. The comprehensive investigations performed on a series of methano-fullerene adducts revealed that $^1O_2$ quantum yields decrease as the double bonds in the fullerene molecule are opened by adduct attachment and the conjugated area of the fullerene core consequently decreases [42,43]. The observed reduction of singlet oxygen production was apparently a consequence of the decrease in both yields and lifetimes of the excited fullerene triplets [42,43]. Interestingly, the opening of just one double bond in the fullerene core following attachment of dihydroxycyclohexano mono-adduct doubled the rate of singlet oxygen quenching by fullerene [44]. It thus appears that the main mechanism for the reduction of $^1O_2$ quantum yield upon fullerene functionalization lies in the diminished capacity of the perturbed fullerene core to generate singlet oxygen, but the increase in $^1O_2$-quenching capacity might also be involved. The complexity of this issue is further emphasized by the fact that some bis- and tris-regioisomers with the same number of identical adducts at different positions display distinct photophysical parameters [35,43,45]. This indicates that the electronic structures governing the photophysical properties of the fullerene derivatives are influenced not only by the structure of addends, but also by the specific addition pattern.

In the absence of intentional photosensitization, many water-soluble fullerenes act as potent deactivators of various ROS, including singlet oxygen, superoxide anion and hydroxyl radicals ($^{\cdot}OH$) [46–49]. This might appear somewhat surprising, having in mind the fairly low $^1O_2$-quenching capacity of pristine C_{60} [6] and the lack of any documented ability for deactivation of other oxygen-centered radicals [17]. While this suggests that covalently attached groups might play an important role in the ROS scavenging by derivatized C_{60}, the dependence of ROS-deactivating capacity of fullerenes on the extent and/or type of functionalization has scarcely been directly assessed. The singlet oxygen-quenching rate constant of bis-malonic C_{60} is two orders of magnitude higher than that of pristine C_{60}, but this increase is difficult to interpret because of the different solvents used [47]. However, the increase by a factor of three of the rate constant for the dendrofullerene compared to

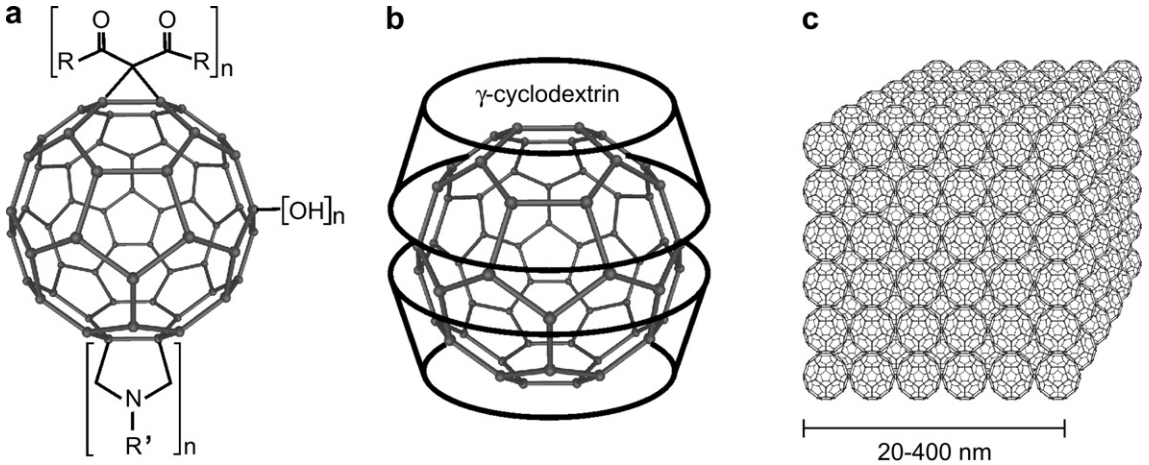

Fig. 3. Schematic representation of different types of C_{60} preparations: (a) water-soluble C_{60} derivatives (R = –OH, NH_2…; R′ = CH_3 or other). For detailed structures, see Bosi et al. [3]; (b) γ-cyclodextrin/C_{60} complex; (c) C_{60} nanocrystals prepared by solvent exchange or sonication/stirring.

the bis-malonic C_{60} could be easily due to the dendrimer's 18 carboxylic acid groups and 8 amino functionalities that are effective $^1O_2$ quenchers [47]. Based on the lifetime of singlet oxygen in a variety of solvents, Hurst and Schuster [50] calculated the $^1O_2$-quenching rate constants for different X–Y bonds, and found that –OH bond should quench $^1O_2$ most efficiently (rate constant 2×10^3 L M^{-1} s^{-1}). While this seems quite low compared to the $^1O_2$-quenching rate constant of pure C_{60} (10^4–10^5 L M^{-1} s^{-1}), the search of the NDRL Radiation chemistry database [51] reveals that $^1O_2$-scavenging capacity of alcohols exponentially increases with the number of –OH groups (rate constants $\approx 10^3$, 10^6 and 10^9 L M^{-1} s^{-1} for some monohydroxy-, dihydroxy- and trihydroxy-alcohols, respectively). Similarly, the $^1O_2$-deactivating capacity of malonic acid [$CH_2(COOH)_2$, rate constant 10^4 L M^{-1} s^{-1}] is one order of magnitude higher than that of acetic acid (CH_3COOH, rate constant 10^3 L M^{-1} s^{-1}). We therefore propose that such unusually high rate of increase in singlet oxygen-quenching capacity might also occur with increasing C_{60} functionalization, thus endowing the highly functionalized fullerenes with excellent ability for $^1O_2$ deactivation.

The above model, however, might not be applicable to other ROS, for example $^{\bullet}OH$. Namely, Guldi and Asmus [52] observed a negative correlation between the $^{\bullet}OH$-quenching rate constants and the number of functionalizing addends in a series of different C_{60} derivatives. This discrepancy is probably related to the different quenching modes of $^1O_2$ vs. $^{\bullet}OH$. While the former is presumably deactivated via physical quenching due to electronic to vibrational energy transfer, the quenching of $^{\bullet}OH$ proceeds through chemical reaction ($^{\bullet}OH + C_{60} = HO–C_{60}$) involving carbon atoms of the fullerene core that are connected with π double bonds. Considering that the number of π bonds in the fullerene moiety becomes smaller with attaching various functional groups, it is expected that the reactivity towards hydroxyl radical addition will decrease with increasing functionalization. Interestingly, the deactivation of superoxide anion by malonic C_{60} derivatives apparently does not occur via stoichiometric "scavenging," as described for the hydroxyl radical, but through catalytic dismutation [48]. This is indicated by the lack of fullerene structural modifications, regeneration of oxygen and production of hydrogen peroxide, all consistent with a catalytic mechanism [48]. The authors proposed a model in which electron-deficient regions on the C_{60} sphere (C_{60}^{+}) work in concert with malonyl groups attached to fullerene core to electrostatically guide and stabilize superoxide, thus promoting its deactivation through following half-reactions: (1) $C_{60}^{+} + O_2^{\bullet -} \rightarrow C_{60} + O_2$; (2) $C_{60} + O_2^{\bullet -} + 2H^+ \rightarrow C_{60}^{+} + H_2O_2$ [47].

Finally, among the most important additional factors that could affect ROS-quenching capacity of some C_{60} derivatives is their property to form nanoscale aggregates with reduced surface-to-volume ratio. This could explain the fact that the antioxidant activity of polyhydroxylated C_{60} (fullerol) in water fails to rise linearly with the concentration, consistent with its tendency to form aggregates whose size sharply rises with increasing concentration [33]. Accordingly, the similar lack of concentration-dependent rise in antioxidant activity was not observed with hexasulfobutyl-C_{60}, another water-soluble derivative forming smaller aggregates that maintain their size in a wide concentration range [33].

3.2. Surfactant-modified C_{60}

Another group of fullerene compounds with possible application in biomedicine is comprised of supramolecular fullerene nanoparticles embedded in surfactants or other solubilizing agents (e.g. cyclodextrins or polymers). The porosity of coating could determine the ability of oxygen dissolved in water to reach fullerene core and, consequently, the rate of reactive oxygen generation upon photosensitization. For example, although the effective shielding of C_{60} within the host molecule in γ-cyclodextrin/C_{60} complex reduces collisional deactivation of excited triplets with ground state molecules and thus provides a remarkably long triplet lifetime, at the same it hinders the contact of C_{60} with molecular oxygen, so the overall ability for $^1O_2$ formation is one order of magnitude lower when compared to that of pristine C_{60} [52]. Moreover, it appears that the chemical properties of the surfactant or solubilizing agent might influence the type of photochemical reaction leading to production of superoxide anion (Type 1) or singlet oxygen (Type 2). While UV- or visible light-excited γ-cyclodextrin/C_{60} complex is a potent generator of singlet oxygen [53,54], photosensitization of C_{60} complexes with polymers such as polyvinyl-pyrrolidone (PVP) or polyethylene-glycol (PEG) mainly results in production of superoxide anion [15,55]. This could be explained by the fact that the excited triplet state of C_{60} is an excellent electron acceptor and, in the presence of donor such as PVP, is easily reduced by electron transfer, resulting in the formation of highly stable charge transfer complex [56]. The fullerene radical anion entrapped in the PVP matrix then transfers one electron to molecular oxygen to produce superoxide, and the reaction can be further enhanced in the presence of other reducing agents, such as NADH, at physiological concentrations found in cells [15].

Both PVP/C_{60} and PEG/C_{60}, as well as γ-cyclodextrin/C_{60} complex, were also reported to efficiently quench superoxide and/or hydroxyl radicals [49]. However, in the case of the γ-cyclodextrin/C_{60} complex, it was found that $^{\bullet}OH$-quenching was mediated completely by macromolecular host [52], which is consistent with the very high rate constant (5×10^9 L M^{-1} s^{-1}) for $^{\bullet}OH$ deactivation by γ-cyclodextrin [57]. Although similar observations have not been reported for other solubilizing agents, it is conceivable that large host molecules could at least partly contribute to antioxidant activity of supramolecular C_{60} complexes, particularly in the case of the surfactants with high ROS-quenching rate constants such as polyvinyl-alcohol (1.5×10^8 L M^{-1} s^{-1} for $^{\bullet}OH$ deactivation) [58] or sodium dodecyl sulfate (SDS; 1×10^9 L M^{-1} s^{-1} for $^1O_2$ deactivation) [51]. Interestingly, we have observed a potent superoxide scavenging activity of SDS-solubilized C_{60}, although surfactant itself at relevant concentrations was only marginally effective (Markovic et al., unpublished data). This might be due to the re-orientation of surfactant's ROS-scavenging groups upon complexation with C_{60}, enabling better contact with reactive oxygen, or some other physico-chemical host–fullerene interaction resulting in a synergistic increase of antioxidant capacity.

3.3. Solvent exchange-solubilized C_{60}

We have recently focused our attention to fullerene nanocrystalline suspension prepared by solvent exchange using tetrahydrofuran (THF). This form of C_{60} appears to be endowed with the unusual ability to produce large amounts of both superoxide and singlet oxygen in ambient light conditions and at extremely low (ppb) concentrations [59–61]. This seems even more unexpected considering the high extent of THF/C_{60} aggregation, which should presumably minimize its photosensitizing power by markedly reducing the active surface area. We have therefore assumed certain degree of porosity of THF/C_{60} nanocrystals, allowing for the diffusion of molecular oxygen into its interior, which is filled with THF entrapped during solvent exchange procedure [62]. Despite the fairly high THF content, estimated to be approximately 10%, the ROS production by THF/C_{60} could not be simply attributed to the solvent, as THF completely failed to produce detectable amounts of reactive oxygen at the relevant concentrations [60,63].

In order to get some insight into the mechanisms responsible for the ROS generation by THF/C_{60}, we have developed a kinetic model to describe singlet oxygen generation by fullerene nanocrystals, based on the model for fullerene solutions [64]. Assuming that the nanocrystal is composed of fullerenes with the property to produce

singlet oxygen and solvent molecules that quench singlet oxygen, we have modeled $^1O_2$ generation by C_{60} nanocrystals impregnated with THF, ethanol (EtOH) or water. The following set of reactions inside nanocrystal is considered: the absorption of light by ground state fullerene and singlet–triplet transition, the decay of triplet fullerene, the generation of $^1O_2$, the quenching of $^1O_2$ by ground state fullerenes, the transition between the state $^1\Sigma$ and $^1\Delta$ in an oxygen molecule, and finally, the quenching of $^1O_2$ by the molecules of the solvent (THF, ethanol or water) intercalated in the fullerene crystalline lattice. These photodynamic reactions are described with kinetic equations [61]. The predicted ability of different C_{60} preparations to generate $^1O_2$ (THF/C_{60} > EtOH/C_{60} > H_2O/C_{60}), which inversely correlates with the $^1O_2$-quenching power of the intercalated solvent (THF < EtOH < H_2O), was confirmed by electron paramagnetic resonance and fluorescence-based measurement of $^1O_2$ production [61]. These data indicate that the $^1O_2$-quenching power of the solvent entrapped within fullerene crystals could determine their overall ability for ROS production. However, regardless of the putative role of intercalated solvent, the mechanisms underlying the unusually high photosensitizing response of THF/C_{60} are still to be explained. Of note, in contrast to data obtained by Sayes et al. [59,60] and us [61,63], Lee et al. [65] failed to demonstrate the ability of THF/C_{60} to produce singlet oxygen or superoxide anion. While this might be related to different methodology used to detect $^1O_2$ and $O_2^{\bullet-}$ in these studies – furfuryl alcohol- and nitroblue tetrazolium-based measurement [65] vs. electron paramagnetic resonance and fluorescence-based detection [59–61,63], the exact reasons responsible for this discrepancy remain to be clarified.

3.4. Pure C_{60} in water

Only few studies have exploited ROS-related behavior of pure C_{60} brought into water through long-term stirring, yielding stable aggregates of nanometer size. Unlike THF/C_{60}, pure C_{60} suspension in water was unable to generate ROS in ambient light conditions [61]. However, pure C_{60} in water readily produced singlet oxygen upon photoexcitation [66], thus confirming that C_{60} prepared in this way retained its photosensitizing capacity. On the other hand, we were unable to demonstrate any ROS-quenching ability of C_{60} water suspension produced by long-term stirring (Markovic et al., unpublished data), which could be due to the fairly low C_{60} concentrations and/or the absence of surfactants with potent antioxidant activity.

A summary of the ROS-generating/quenching properties of water-soluble C_{60} derivatives and nanoparticles is given in Table 1.

4. ROS-dependent biological effects of C_{60}

An important feature of various C_{60} preparations that facilitates their biological reactivity is the ability to penetrate cell membrane and gain access to cell cytoplasm, organelles and nucleus, as predicted by theoretical studies and confirmed in various experimental settings [67–71]. Singlet oxygen, generated by energy transfer from C_{60} to molecular oxygen (Type II photochemical mechanism), could directly react with various biological targets, including lipids, proteins, nucleic acids and carbohydrates, resulting in cell damage or functional alterations [8]. In a complex environment such as intracellular space, which contains reducing agents (e.g. NADH), the electron transfer from the fullerene radical anion to molecular oxygen (Type I photochemical mechanism) or directly from the reducing agent to $^1O_2$ could also yield superoxide [15,72,73] and, subsequently, hydrogen peroxide and hydroxyl radical (Fig. 4). These biologically important O_2 derivatives, owing to their redox potential, can modulate cell death, proliferation, migration, inflammation, secretion, extracellular matrix protein synthesis, neurotransmission and many other functions in various cell types [74–76]. It is therefore a plausible strategy to employ ROS-generating/quenching capacity of C_{60} in designing biologically active agents with diverse functions.

4.1. Toxicity

The assessment of C_{60} toxicity is an absolute and obvious prerequisite for its potential use in biomedicine and consumer products. Also, the interactions of C_{60} with water ecosystems represent an important aspect of its industrial mass-production, particularly in view of the possible unintentional generation of fullerene aggregates in aqueous environments. These concerns seem justified, as C_{60} can be a potent source of ROS, and it is well known that an

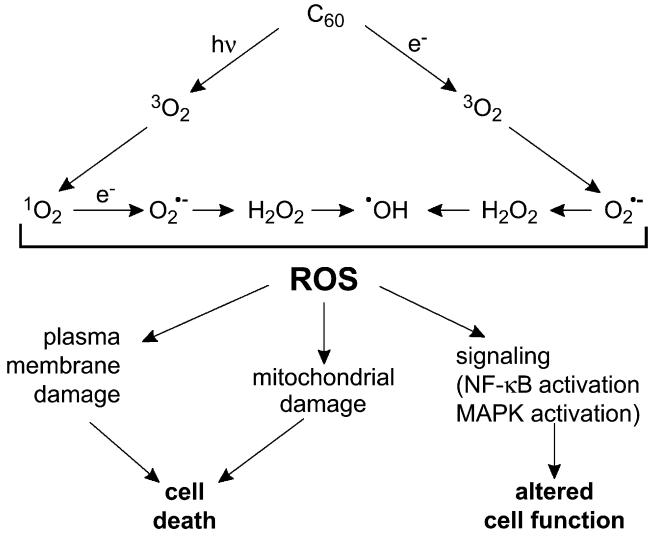

Fig. 4. Fullerene-mediated generation of various types of ROS in biological environment and their possible effects (NF-κB – nuclear factor-κB; MAPK – mitogen-activated protein kinase).

Table 1
An overview of the ROS-generating/quenching properties of different C_{60} preparations

Fullerene type	ROS generation		ROS quenching		Factors affecting ROS generation/quenching
	Type of ROS	Capacity	Type of ROS	Capacity	
Derivatized	$^1O_2$, $O_2^{\bullet-}$	↓	$^1O_2$, $O_2^{\bullet-}$, $^\bullet OH$	↑	Number and type of functional groups
Surfactant coated	$^1O_2$ (cyclodextrin), $O_2^{\bullet-}$ (PVP, PEG)	↓	$O_2^{\bullet-}$, $^\bullet OH$	↑	Type of surfactant; agglomeration
Solvent exchange prepared	$^1O_2$, $O_2^{\bullet-}$ (THF)	↑ (THF)[a] ↓ (ethanol)[a]	n.a.	n.a.	Type of solvent; agglomeration
Water stirred	$^1O_2$	↓	n.a.	n.a.	Agglomeration

ROS-generating/quenching capacity refers to pure C_{60} (n.a. – not assessed).
[a] Not directly compared, no rate constant available.

excessive ROS generation that overwhelms antioxidant capacity results in oxidative stress and significant damage to the cell in the form of cytotoxic or mutational outcome [77].

In accordance with the inability of C_{60} to produce ROS unless exposed to a strong light source, many classical toxicity studies have failed to demonstrate significant acute or sub-acute in vivo toxicity, genotoxicity or in vitro cytotoxicity of various C_{60} preparations applied at relatively large concentrations [78–85]. However, the perception of C_{60} as relatively non-toxic contradicts the surprising demonstration by Sayes et el. [59] that solvent exchange-prepared non-derivatized C_{60} nanoparticles (THF/C_{60}), unlike water-soluble derivatives, can generate high amount of ROS and kill human cells at extremely low (ppb) concentrations, even if exposed only to ambient light during preparation/cell treatment (dark cytotoxicity). The same group subsequently reported that cytotoxic activity of THF/C_{60} was mediated through ROS-mediated cell membrane lipid peroxidation [60], which was consistent with the increase in lipid peroxidation observed in rat liver microsomes treated with pure C_{60} [86], or in the fish brains following exposure to C_{60} nanocrystals [87]. Sayes et al. have proposed that the extremely high, cytotoxic ROS production by THF/C_{60} could be an inherent property of pure, underivatized C_{60}, corroborating their hypothesis by showing that ROS generation and cytotoxicity decrease with increasing derivatization of the fullerene cage [59]. This was consistent with the previous reports that covalent attachment of various functional groups to the fullerene core changes the photophysical properties of C_{60} and decreases its capacity for $^1O_2$ generation [35,42,43]. Accordingly, by directly comparing the ROS-generating/cytotoxic capacity of THF-prepared and polyhydroxylated C_{60}, we have confirmed that cytotoxicity of the former was mediated by intracellular ROS accumulation that could be prevented by antioxidant treatment, while marginal toxicity of the latter was ROS-independent [88].

While these results raised the awareness about potential toxicity of pristine C_{60}, an alternative theory suggests that ROS-mediated cytotoxicity of THF/C_{60} could actually stem from the residual presence of THF, which remains intercalated into its lattice [62]. In support of the latter assumption, pure C_{60} suspension prepared by long-term stirring in water was significantly less toxic to mammalian cells in culture and various aquatic organisms [31,61,89], and injection of detergent-solubilized pure C_{60} to rats was tolerated without any signs of acute toxicity [90]. Moreover, the products of THF decomposition (e.g. γ-butyrolactone) occurring in the course of THF/C_{60} preparation have been found partly responsible for the changes in gene expression and reduced survival of larval zebrafish exposed to these fullerene nanoparticles [91]. On the other hand, it appears that the in vitro ROS-mediated cytotoxicity of THF/C_{60} towards mammalian cells could not be simply attributed to the degradation products of THF. Namely, the experimental and mathematical modeling data indicate that the presence of intact THF within C_{60} nanocrystals is necessary for the ROS-mediated cytotoxicity [61,63], presumably due to a fairly long lifetime of singlet oxygen in THF, thus allowing its diffusion to the surface of nanocrystal and subsequent reactivity with the surrounding biomolecules [61]. Accordingly, C_{60} nanoparticles containing solvents (e.g. ethanol or water) with better $^1O_2$-quenching ability than THF cause markedly lower intracellular ROS accumulation and cytotoxicity [61].

Regardless of the complex mechanisms responsible for the ROS generation (see Section 3.3) and the resultant toxicity of THF-prepared C_{60}, it is clear that they depend on the presence of the solvent. While this obviously limits the biomedical potential of THF/C_{60}, it seems that the fears about its possible ecotoxicity have been rather exaggerated, as it is difficult to imagine a scenario resulting in an unintentional generation of THF/C_{60} in aqueous ecosystems. Moreover, in contrast to in vitro data, intratracheal administration of THF/C_{60} to rats did not result in significant acute pulmonary toxicity [92]. Nevertheless, in spite of fairly compelling evidence for the relative biosafety of C_{60}, it would be unwise to underestimate its potentially toxic effects. Namely, it has been reported that C_{60} nanoparticles prepared by extended mixing in water and some water-soluble C_{60} derivatives can still display toxic or mutagenic effects in various experimental systems [20,87,93–100]. This could be related to the use of a particularly sensitive target cell type, such as endothelial or ocular lens cells [93,94], extremely high concentrations of C_{60} [20,95,96], different route of injection [95], increase in toxicity upon attachment of certain functional groups [97], interaction with the toxic modifying compound or other contaminants [98,99], or a specific methodology for evaluating mutagenicity [100]. While the role of ROS in these toxic effects was not investigated, it should be noted that they were observed in the absence of overt photosensitization. It is therefore possible that C_{60} might exert ROS-independent toxicity, or that some C_{60} preparations (e.g. THF/C_{60}) might be more sensitive to photoexcitation and able to produce ROS if exposed to ambient light during preparation and/or cell treatment. One of the mechanisms for the distinct cytotoxic potency of various C_{60} derivatives might lie in their different ability to interact with cell membrane and gain access to intracellular space. Namely, it has been shown that toxic cationic derivatives with high hydrophobic/hydrophilic surface area ratio penetrate cell membrane more easily than non-toxic neutral and anionic derivatives [20].

4.2. Anticancer and antimicrobial activity

The potent ability of fullerenes to photosensitize transition of molecular oxygen to highly reactive ROS makes them promising candidates for the photodynamic killing of cancer cells. The main advantage of this therapeutic approach is selectivity, achieved by tumor-specific activation of photosensitizing agent by highly focused light beam delivered to tumor region at the surface of the body or to internal tumors using optical fibers [101]. There are many studies demonstrating the efficient photodynamic action of various water-soluble C_{60} derivatives against different types of cultured cancer cell lines (cervical, larynx, lung and colon carcinoma) and malignant tumors in vivo (reviewed in Refs. [102]). A particularly promising approach involves linkage of fullerenes with other photosensitizers, such as porphyrin, exploiting the unique photophysical and redox properties that endow these C_{60}–porphyrin dyads with extremely high capacity for ROS-mediated cytotoxicity even in the relative absence of oxygen [103]. The observed anticancer activity of fullerene derivatives was apparently dependent on generation of both singlet oxygen and superoxide anion [103,104], and it was inversely correlated with the extent of derivatization of the fullerene cage [104,105]. The latter is consistent with the reduction of the fullerene's ROS-generating capacity that occurs upon increasing the number of covalently attached functional groups [35,42,43]. Moreover, a closer examination of the structure–activity relationship reveals that C_{60} derivatives containing more potent $^1O_2$-quenching groups (e.g. –OH) display lower photodynamic activity compared to those containing the same number of groups with inferior $^1O_2$-quenching ability (e.g. –CH) [104]. This agrees with the assumption that overall ROS production by a C_{60} derivative is in part determined by the ability of its functional groups to deactivate C_{60}-generated ROS (see Section 3.1). However, some sugar-pendant derivatives displayed different photodynamic efficiencies despite similar production of $^1O_2$ [39], while tris-malonic acid C_{60} was more photocytotoxic than monoadduct in spite of the higher $^1O_2$ quantum yield for the latter [106]. These data suggest that, in addition to $^1O_2$-producing capacity, other factors, such as degree of cell membrane incorporation and cellular uptake, might profoundly influence the phototoxicity of C_{60}-based agents.

The photodynamic antitumor action of water-soluble C_{60} derivatives apparently involves induction of the "programmed" cell death (Type I), known as apoptosis [103,104]. This type of cell demise is characterized by activation of the caspase enzyme family and fragmentation of DNA, which occurs without plasma membrane breakdown and is followed by recognition and removal of apoptotic cell by phagocytes in the absence of inflammation [107]. This is consistent with the preferential mitochondrial localization of water-soluble C_{60} derivatives [67,68], having in mind that ROS-induced mitochondrial dysfunction is a key initial step in the "mitochondrial" pathway of apoptosis [107]. Interestingly, polyhydroxylated C_{60} was able to suppress proliferation and induce apoptosis of tumor cells in the absence of photosensitization and ROS production [88,108,109]. In view of the involvement of redox-sensitive transcription factors, such as NF-κB, in regulation of cell growth and apoptosis [110], these results indicate an interesting possibility that C_{60} could exert its antiproliferative/pro-apoptotic action not only by producing cell-damaging ROS, but also through antioxidant effects.

Unlike water-soluble C_{60} derivatives, C_{60} nanoparticles prepared by addition of conventional surfactants (e.g. SDS, Tween) or polymers (e.g. PEG, PVP) have been only sporadically tested for their photodynamic activity against cancer. This might seem somewhat surprising, as non-derivatized C_{60} displays higher $^1O_2$ quantum yield in comparison with functionalized water-soluble derivatives [35,42,43], so it should be a more efficient photosensitizer. Moreover, the relatively large size of these C_{60} nanoparticles (up to several hundreds of nm) should presumably provide high intratumor concentration through "enhanced permeability and retention" effect [111], due to abnormally large vascular pores and impaired lymphatic drainage in tumors. Indeed, PEG/C_{60} conjugate exhibited higher accumulation and more prolonged retention in the tumor tissue than in normal tissues, showing a stronger tumor-suppressive photodynamic effect than conventional photosensitizer Photofrin [112].

Interestingly, the potent ROS-dependent anticancer activity of another nanoparticulate C_{60} preparation, solvent exchange-prepared THF/C_{60}, was readily initiated at low-level ambient light and could not be further stimulated by either visible or UV light [59,88]. The observed effect was oxidative stress-mediated and, in contrast to pro-apoptotic action of water-soluble C_{60} derivatives, involved "accidental" cell death – necrosis [61,63,88]. This type of cell death, unlike apoptosis, is typified by vacuolation of the cytoplasm, breakdown of the plasma membrane and release of cellular contents, resulting in the induction of inflammatory response [107]. The apparent discrepancy regarding the mechanisms of cell death (necrosis vs. apoptosis) could stem from the extremely high ROS production by THF/C_{60}, leading to rapid lipid peroxidation and permeabilization of cell membrane [60,63,88], which is consistent with mainly cell membrane vs. mitochondrial accumulation of nanoparticulate vs. water-soluble C_{60} [60,67–70]. However, some amount of THF/C_{60} probably gained access to cell cytoplasm, as indicated by its ability to influence certain intracellular events involved in necrosis induction, such as activation of mitogen-activated protein kinases and mitochondrial depolarization [61,63]. In view of the immunostimulatory properties of necrotic cells and resistance of tumor cells to apoptosis, it has been proposed that necrosis might be more efficient than apoptosis in inducing tumor regression [107]. On the other hand, it is more difficult to restrict necrosis to tumors, and THF/C_{60} was indeed highly toxic to a variety of normal mammalian cells [59,63]. Nevertheless, it seems conceivable that the large size of THF/C_{60}, which could be easily controlled during preparation [28], might afford in vivo tumor-selectivity through "enhanced permeability and retention" effect. Accordingly, using mouse B16 melanoma model, we have observed that intraperitoneally injected THF/C_{60} accumulates more in melanoma cells than in normal tissues (Trajkovic et al., unpublished data). In a different approach to selective tumor targeting with C_{60} nanoparticles, we have demonstrated that noncytotoxic concentrations of THF/C_{60} and anticancer cytokine tumor necrosis factor (TNF) synergize in inducing oxidative stress and death of TNF-sensitive cancer cells, without harming normal cells [113]. Moreover, it appears that THF/C_{60}, at low doses that do not trigger oxidative stress, might still affect tumor cells by inducing cell cycle arrest and autophagy (programmed cell death Type II) [114], a process of self-cannibalization during which cells digest their own proteins through a lysosomal degradation pathway [107]. While the exact mechanisms underlying these ROS-independent effects are still to be revealed, they are consistent with the ability of C_{60} nanoparticles to gain access to cell cytoplasm, as indicated by theoretical models and demonstrated in the cellular uptake experiments [67–71]. Importantly, the observed oxidative stress-independent actions of THF/C_{60} were apparently selective for tumor cells, leaving their non-transformed counterparts mainly unaffected [114].

In addition to killing tumor cells, C_{60} could be also used for the photodynamic inactivation of bacteria, as convincingly demonstrated in studies examining the effects of water-soluble and nanoparticulate C_{60} on various bacterial strains [102]. The effects were significantly more pronounced in Gram positive (*Staphylococcus spp., Streptococcus spp.*) than in Gram negative bacteria (*Klebsiella Pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Streptococcus pyogenes*) [115], indicating that the bactericidal action was dependent on the fullerene insertion into the microbial cell wall, the structure of which differs between Gram positive and Gram negative bacteria. Accordingly, the antibacterial action of C_{60} nanoparticles was associated with alterations in membrane lipid composition and membrane fluidity in response to oxidative stress [116], and cationic C_{60} derivatives were more effective than neutrally or negatively charged ones, presumably as a result of the stronger interaction with bacterial cell wall [117]. Moreover, some bis-cationic and tris-cationic fullerenes performed significantly better than classical antimicrobial photosensitizer toluidine blue, under conditions in which mammalian cells were comparatively unharmed [118]. Fullerenes can also serve as building blocks in the solid phase peptide synthesis preparation of antibacterial peptides, resulting in production of fulleropeptides with higher antimicrobial activity than parental compounds [119]. In addition to antibacterial effects, both pure sonicated C_{60} and its derivatives have been reported to inactivate viruses belonging to *Togaviridae*, *Rhabdoviridae* and *Flaviviridae* families, as well as some bacteriophages, through either photodynamic ROS-dependent or ROS-independent actions [66,120,121]. One of the potentially useful ROS-independent actions is the inhibition of HIV replication through incorporation of specifically tailored C_{60} derivatives into the active site of HIV protease [122].

4.3. Antioxidant/cytoprotective activity

Oxidative stress and associated oxidative damage are mediators of cellular injury in many pathological conditions, including autoimmunity, atherosclerosis, diabetes and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and HIV-associated dementia [123]. The exceptionally harmful effects of oxidative stress in neurons are primarily a result of the diminished capacity of the central nervous system to prevent oxidative damage. Mitochondria appear to be both the major source and the target of intracellular ROS [124]. Excess ROS could cause mitochondrial depolarization associated with the opening of mitochondrial permeability transition pore and subsequent release of small pro-apoptotic molecules such as cytochrome c, leading to activation of caspase cascades and apoptosis. In turn, the collapse of the mitochondrial membrane potential

triggers an increase in ROS generation by the electron transfer chain, thus providing a positive feedback mechanism for enhanced ROS production leading to further mitochondrial and cellular injury. Thus, therapies targeted against ROS hold great promise for minimizing cellular injury in oxidative stress-mediated neurodegeneration and other ROS-dependent disorders.

Because of their ability to associate with mitochondria and to quench ROS more efficiently than conventional antioxidants, water-soluble fullerenes such as polyhydroxylated C_{60} (fullerol) or malonic acid (carboxy) derivatives of C_{60} are plausible candidates for cytoprotective antioxidant treatment. With the few exceptions [125–127], most of the studies on cytoprotective action of water-soluble fullerenes were performed with fullerols or carboxyfullerenes. However, because of the high accumulation and retention of fullerol in bone tissues [128] and the absence of a reliable method for scalable production of derivatives with defined number of hydroxyl adducts, carboxyfullerenes might be more plausible candidates for biomedical application than fullerol. Both fullerol and carboxyfullerenes reduced ROS-dependent neuronal death induced by engagement of glutamate receptors or K^+ deficiency [129–132], while carboxy-C_{60} was also efficient in protecting dopaminergic neurons from neurotoxin-mediated oxidative stress [133]. In vivo neuroprotective effect of carboxyfullerenes was documented in the amyotrophic lateral sclerosis model in SOD1-mutant mice [130], ischemia-reperfusion-induced cortical infarction [134], and iron- or neurotoxin-induced death of dopaminergic neurons in Parkinson's disease model in rats [135,136]. In accordance with the proposed role of ROS in the nervous system aging, chronic treatment with carboxyfullerenes not only reduced age-associated oxidative stress in the brain, but also significantly extended lifespan and prevented age-related cognitive impairment in mice [137]. Treatment with fullerol also protected rat lungs from ischemia-reperfusion injury [109] and reduced cell death triggered by proinflammatory cytokine TNF, hydrogen peroxide or nitric oxide (NO) [88,113], while carboxy-C_{60} protected ionizing radiation-exposed zebrafish embryos [138] and blocked the oxidative stress-mediated apoptosis induced in various cell types by UV light or cytokines [68,113,139,140]. Surprisingly, C3 regioisomer of tris-malonic C_{60} derivative was more efficient cytoprotective agent than corresponding D3 isomer, despite their similar ability for ROS deactivation and probably due to the better incorporation of the C3 isomer into the lipid membranes [130,140]. This interesting observation indicates that the overall cytoprotective activity of C_{60} derivatives will not only depend on their ROS-scavenging capacity, but also on the strength of their interaction with cellular membranes. In addition to deactivation of classical ROS, it has recently been reported that fullerol can also directly quench nitric oxide (NO) [141], a nitrogen derivative of O_2 produced by NO synthase family of enzymes and displaying a variety of important biological functions, including vasodilatation, neuromodulation, and at high concentrations, cytotoxicity [142]. Moreover, tris-malonyl C_{60} has been found to inhibit the catalytic activity of all three NO synthase isoforms – neuronal, endothelial and inducible [143]. While it has been shown that carboxy-C_{60} can block low dose NO-triggered vasodilatation [144], the ability of fullerol to prevent cell death induced by high concentrations of NO has also been demonstrated [88,109].

The mechanisms underlying ROS deactivation-dependent cytoprotective action of fullerol and carboxyfullerenes mainly involved interference with the oxidative stress-mediated induction of apoptotic cascade. Fullerene treatment efficiently reduced TNF- or UV-triggered activation of caspases [68,113], the key enzymes responsible for initiation of DNA fragmentation during apoptosis [107]. The early pro-apoptotic events, including clustering of apoptosis-inducing receptor Fas, down-regulation of the apoptotic inhibitors survivin and livin, activation/up-regulation of the pro-apoptotic proteins Bid and Bad, as well as mitochondrial depolarization and mitochondrial release of the caspase activator cytochrome C, were all reduced by water-soluble fullerenes [68,109,113,139,145].

In contrast to water-soluble C_{60} derivatives, fullerene nanoparticles prepared from pure C_{60} have rarely been directly assessed for their cytoprotective properties. While it has been reported that polymer (PVP, PEG)- or γ-cyclodextrin-solubilized C_{60} can reduce intracellular ROS levels and ROS production in the cell-free systems [49,146], only the cytoprotective ability of PVP/C_{60} was confirmed in a model of UV-induced death of human skin keratinocytes [147]. Importantly, PVP/C_{60} did not show visible light-induced toxicity towards keratinocytes, indicating its possible usefulness in the prevention of UV skin-injuries. Moreover, PVP/C_{60} reduced articular cartilage degeneration in a rabbit model of osteoarthritis and suppressed catabolic stress-induced production of extracellular matrix-degrading enzymes (matrix metalloproteinases) and apoptosis in human chondrocytes in vitro [148]. Polyoxyethylene sorbitan monostearate (Tween 60)-coated C_{60} was very efficient in preventing oxidative stress-dependent liver damage induced by carboxytetrachloride administration to rats [89], but the mechanisms of the observed in vivo hepatocyte protection were not investigated in more detail. Surprisingly, cytotoxic ROS-generating THF/C_{60} nanoparticles exposed to γ-radiation not only lost their cytotoxic properties, but also acquired the ability to scavenge ROS and protect mammalian cells from oxidative stress-induced death [63]. Collectively, these data indicate that cytoprotection is not restricted to derivatized C_{60}, and that pure C_{60} introduced to water either by surfactant coating or solvent exchange can also display potentially useful cytoprotective features. While it has been observed that different C_{60} nanoparticles (PVP, PEG or γ-cyclodextrin-modified) display different ROS-scavenging properties in terms of the quenching capacity and the type of ROS targeted [49,145], it has not been assessed whether these differences could influence their cytoprotective potency.

4.4. Immunomodulation and other applications

In addition to crucial role in cell death/survival, ROS is emerging as key effectors in signal transduction in various cell types, particularly those involved in immunity against microbes and tumor cells, but also in self-tissue destruction in chronic inflammatory disorders such as autoimmunity and allergy [123]. ROS-dependent intracellular signaling appears to be mediated mainly by activation of the mitogen-activated protein kinase cascade and transcription factor NF-κB [110]. Because of the key roles that these signaling pathways play in activation of macrophages, lymphocytes and other immune cells [110], ROS-modulating fullerene-based agents are potential candidates for developing new therapies for the modulation of immunity in chronic inflammatory conditions, infection and cancer.

Although the classic therapies of cancer and infection are mainly based on therapeutic agents or procedures that directly kill cancer cells and microbes, the enhancement of immune function could be an important auxiliary treatment in these conditions. The in vivo anticancer effect of multihydroxylated metallofullerene containing gadolinium [Gd@$C_{82}(OH)_x$] was associated with the increased accumulation of immune cells within tumor [149]. As the intratumor presence of [Gd@$C_{82}(OH)_x$] was almost negligible and the metallofullerene was unable to kill tumor cells in vitro, it appears that the observed inhibitory effect on tumor growth in vivo was mainly mediated through activation of anticancer immunity. Similarly, it has been demonstrated that the protection from lethal *S. pyogenes* infection by carboxyfullerenes was not only a consequence of a direct inhibition of bacterial growth, but apparently involved the enhancement of the bactericidal activity of neutrophils as well [150]. These effects were consistent with the in vitro stimulatory

Table 2
An overview of the main ROS-mediated biological effects of different C_{60} preparations

Fullerene type		Experimental system	ROS	Biological action(s)	Ref.
Water-soluble C_{60} derivatives	$C_{60}(OH)_n$	Excitotoxic and apoptotic neuronal death in vitro	↓	Neuroprotection	[129,132]
		Nitric oxide or H_2O_2 cytotoxicity in vitro	↓	Prevention of cell death	[88,109]
		Ischemia-reperfusion lung injury	↓	Reduction of vascular damage	[109]
		TNF cytotoxicity in vitro	↓	Inhibition of mitochondrial depolarization, caspase activation and apoptotic cell death	[113]
		In vitro treatment of human lens epithelial cells	↑ PS	Cytotoxicity (apoptosis)	[94]
		Bacteriophage treatment	↑ PS	Bacteriophage inactivation	[121]
		Treatment of mastocytes and basophils in vitro; in vivo model of anaphylaxis	↓	Inhibition of histamine release in vitro and in vivo	[153]
	Malonic acid derivatives	In vitro culture of cancer cells	↑ PS	Cell cycle block, growth suppression	[105,106]
		UV- or TGF-β-induced apoptosis of keratinocytes and hepatocytes	↓	Reduction of mitochondrial depolarization, caspase activation and apoptotic cell death	[68,139,140]
		Excitotoxic and apoptotic neuronal death in vitro	↓	Neuroprotection	[130,131,133]
		Animal models of ALS, Parkinson's disease, cortical infarction, radiation-induced brain damage and nervous system aging	↓	Neuroprotection, improved survival	[130,134,136–138]
	Sulfobytylated C_{60}	Focal cerebral ischemia in rats	↓	Neuroprotection	[125]
	Pyrrolidinium derivatives	In vitro culture of cancer cells	↑ PS	Cancer cell death (apoptosis)	[104]
		In vitro treatment of bacteria	↑ PS	Bacterial killing	[118]
	C_{60}-porphyrin	In vitro culture of cancer cells	↑ PS	Cancer cell death (apoptosis)	[103]
	Sugar derivatives	In vitro culture of cancer cells	↑ PS	Cancer cell death	[39]
Surfactant-coated, solvent exchange- or sonication-prepared C_{60} nanoparticles	PVP/C_{60}	UV-induced death of keratinocytes	↓	Prevention of cell death	[147]
		Chondrocyte apoptosis in vitro; animal model of osteoarthritis	↓	Reduction of chondrocyte apoptosis in vitro and cartilage destruction in vivo	[148]
	PEG/C_{60}	In vivo treatment of cancer	↑ PS	Tumor regression	[55,112]
	$Cyclodextrin/C_{60}$	Incorporation into rat liver microsomes	↑ PS	Lipid peroxidation	[53]
	$Tween/C_{60}$	Toxin-induced liver damage	↓	Liver protection	[90]
	THF/C_{60}	In vitro culture of normal and cancer cells	↑	Membrane lipid peroxidation, mitochondrial depolarization, MAPK activation, cell death (necrosis)	[59–61,63,88]
		In vitro TNF treatment of cancer cells	↑	Mitochondrial depolarization, caspase activation, apoptosis/necrosis	[113]
		In vitro culture of normal and cancer cells	↑	Cell cycle block, autophagy; normal cells less affected	[114]
		In vivo treatment of fish	↑	Oxidative brain damage, death	[87]
	Sonicated C_{60}	In vitro treatment of viruses	↑ PS	Virus inactivation	[66]

The effects on ROS generation were directly assessed, except in the case of some in vivo studies, where they were indirectly inferred. For an extensive review of photodynamic activity of fullerenes, see Mroz et al. [102]. (PS – photosensitization; TNF – tumor necrosis factor; TGF – transforming growth factor; ALS – amyotrophic lateral sclerosis; MAPK – mitogen-activated protein kinase).

action of some C_{60} derivatives on the secretion of proinflammatory cytokines TNF, IL-1 and IL-8 in cultures of human keratinocytes [151]. On the other hand, immune-mediated inflammation in response to infection can be harmful to oxidative stress-sensitive tissues such as brain. Intraperitoneal injection of carboxyfullerene completely protected mice from bacteria (*E. coli*)-induced lethal meningitis, while in the same conditions the classic antiinflammatory drug dexamethasone was only partly efficient [152]. Interestingly, the observed effect was not due to a direct antibacterial activity of C_{60}, but resulted from fullerene-mediated

interference with the secretion of neurotoxic proinflammatory mediators TNF and IL-1 and reduction of the increase in blood–brain barrier permeability and inflammatory neutrophilic infiltration [152]. Polyhydroxylated C_{60} and *N*-ethyl-polyamino C_{60} prevented the in vivo release of histamine and drop in body temperature in a mouse model of anaphylaxis [153]. The anti-allergic effect was associated with the ability of fullerenes to block IgE-induced elevation of ROS in human mast cells, which resulted in the inhibition of signaling molecules involved in release of allergic mediators [153]. While these results encourage further exploration of fullerenes as immunomodulating agents, the main question that remains to be answered is what are the mechanisms responsible for their ability to either stimulate or suppress immune functions in different experimental settings? More specifically, the role of ROS in the fullerene-mediated immunomodulation needs to be examined more closely.

In addition to the effects described here and summarized in Table 2, fullerenes have displayed other potentially useful biological actions. For example, fullerol was more efficient than classic antioxidant ascorbic acid in reducing the proliferative responses of vascular smooth muscle cells in culture [154]. The observed antiproliferative effect of fullerol was probably mediated through suppression of protein kinase C and protein tyrosine kinase activity [154], indicating possible usefulness of C_{60}-based agents in restricting vascular smooth muscle proliferation in atherosclerosis and post-angioplasty restenosis. Besides enzyme inhibition, fullerenes are promising candidates for many other biomedical applications, such as drug delivery [155], gene transfection [156], diagnostic imaging [157] and design of various composite biomaterials [158]. Since the effects of C_{60} in these applications are not directly mediated by ROS production/quenching, their analysis is beyond the scope of the present review. Nevertheless, it should be born in mind that biocompatibility and performance of fullerenes in these applications might be significantly influenced by their ROS-generating/quenching profile.

5. Concluding remarks

Despite great enthusiasm about the potential use of fullerenes in biomedicine, the progress in their development as plausible drug candidates is still in its infancy. One of the main reasons for this, in addition to the lack of methods for large-scale synthesis of highly purified preparations compatible with pharmaceutical applications, lies in our inadequate understanding of structure–function relationships with respect to various modifications required to provide water solubility. The ability to produce or quench highly reactive ROS, the feature that mediates most of the fullerenes' biological and toxic actions, is particularly sensitive to a covalent attachment of functional groups to the fullerene core, as well as to the physico-chemical changes introduced by solubilization procedures for the preparation of fullerene nanoparticles. The ROS-producing/quenching ability of covalently functionalized water-soluble fullerenes mainly depends on the number, structure and substitution pattern of the addends, and may significantly vary with the type of ROS produced/quenched ($^1O_2$, $O_2^{\bullet-}$ or $^{\bullet}OH$). On the other hand, the type of the solubilizing agent markedly affects ROS-related behavior of nanoparticulate C_{60} composites, either directly (through inherent ROS-quenching ability) or indirectly (by determining the surface area, degree of agglomeration and surface chemistry). Moreover, the capacity for ROS generation/quenching in certain cases appears to synergistically increase as a result of some unusual interactions of the fullerenes in molecular or nanoparticulate form with the functional groups (e.g. –COOH, enabling SOD-mimetic activity) or modifying agents (e.g. THF, providing high cytotoxicity). These complex properties of C_{60} preparations shape distinct ROS-mediated biological effects that are further fine-tuned by the extent of membrane incorporation, cellular internalization and localization in different intracellular compartments. The full exploration of these relations in order to tightly control ROS-dependent biological effects of different fullerene preparations clearly represents one of the principal goals in their potential development for therapeutic use.

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