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Carbon nanomaterials: Biologically active fullerene derivatives

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SUMMARY
Since their discovery, fullerenes, carbon nanotubes, and graphene attract significant attention of researches in various scientific fields including biomedicine. Nano-scale size and a possibility for diverse surface modifications allow carbon nanoallotropes to become an indispensable nanostructured material in nanotechnologies, including nanomedicine. Manipulation of surface chemistry has created diverse populations of water-soluble derivatives of fullerenes, which exhibit different behaviors. Both non-derivatized and derivatized fullerenes show various biological activities. Cellular processes that underline their toxicity are oxidative, genotoxic, and cytotoxic responses. The antioxidant/cytoprotective properties of fullerenes and derivatives have been considered in the prevention of organ oxidative damage and treatment. The same unique physiochemical properties of nanomaterials may also be associated with potential health hazards. Non-biodegradability and toxicity of carbon nanoparticles still remain a great concern in the area of biomedical application. In this review, we report on basic physical and chemical properties of carbon nano-clusters – fullerenes, nanotubes, and graphene – their specificities, activities, and potential application in biological systems. Special emphasis is given to our most important results obtained *in vitro* and *in vivo* using polyhydroxylated fullerene derivative $C_{60}(OH)_{24}$.
Keywords: fullerene C_{60}; fullerenol; nanoparticles; free radical scavengers; cell line; cytotoxicity

INTRODUCTION

Thirty years ago the third allotropic modification of carbon, fullerene, was discovered [1]. Until then two forms of carbon were known – graphite and diamond. Some years later, in 1991, carbon nanotubes were characterized, and in 2004 the third form of "new carbon," graphene, was isolated from graphite by Andre Geim and Kostya Novoselov [2, 3]. Since their discovery, carbon nanoallotropes attract significant attention of researches in various scientific fields including biomedicine. Fullerenes are entirely insoluble in water and polar solvents. Several fundamental properties of fullerenes allow their functionalization, which makes them interesting for biological research. Water-soluble fullerene derivatives may effectively interact with DNA, proteins, and living cells. Biological activities of fullerenes are diverse. They induce oxidative, genotoxic, and cytotoxic response, while their antioxidative/cytoprotective properties have been considered for utilization in treatment of oxidant-mediated diseases [4]. One of the first reports on biological activity of soluble fullerene derivatives referred to inhibition of HIV-1 protease and since than a variety of biological activities of fullerene derivatives have been reported [5, 6]. Here, we briefly report on basic physical and chemical properties of carbon nano-clusters – fullerenes, nanotubes, and graphene – their specificities, activities, potential application in biological systems, and their capacity to harm both human health and the environment. Special emphasis will be given to our most important results obtained *in vitro* and *in vivo* using polyhydroxylated fullerene derivative $C_{60}(OH)_{24}$.

DISCOVERY AND PHYSICAL AND CHEMICAL PROPERTIES OF CARBON NANOMATERIALS

Long before experimental evidence and synthesis of fullerenes C_{60}, has been a hypothesis that hollow carbon cage may exist. In 1970, Yoshida and Osawa suggested a spherical structure of an icosahedron for the C_{60}; Iijima and Huffman-Kreatschmer team could not recognize this molecule in their experimental data; in 1972, Russian chemists Botchvar and Gal'pern predicted the existence of C_{60} based on physicochemical calculations but their work remained unnoticed [7]. Croto and Smolly "rediscovered" C_{60} accidentally during their experiments, and together with Curl they were rewarded with the 1996 Nobel Prize in Chemistry [1]. The name "fullerenes" to the family of carbon clusters was given by Croto and Smolley after American futuristic architect, philosopher, and writer Richard Buckminster Fuller, who designed geodesic dome in which a pentagon was used to provide curvature. This new form of carbon is reminiscent of a soccer ball and in the older literature spherical C_{60} was referred to as "footballene" or "buckyball", and

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the hollow cylindrical carbon was named "buckytubes/nanotubes".

Fullerene can be found in meteorites. Krätschmer et al. [8] first synthesized a macroscopic quantity of fullerene in 1990, and today tones of fullerene material are produced per year using different processes.

Fullerenes (Cn 540 ≥ n ≥ 20) are defined as polyhedral closed cages made up entirely of three-coordinate carbon atoms. They contain 12 pentagons and different number of hexagons. The most known is spherical fullerene C_{60}, which contains 12 pentagons and 20 hexagons. It has geometry of truncated icosahedrons. Carbon atoms in C_{60} are located in the vertices of regular truncated icosahedrons; all atoms are equivalent giving a single peak (δ = 144.3 ppm) in its ^{13}C NMR spectrum. Diameter of C_{60} is 0.710 ± 0.007 nm and the inner diameter of electron cloud is estimated at ~ 0.35 nm. C_{60} is considered the most perfect symmetry form existing in nature [1].

Pure fullerenes are black crystals or powder. Fullerenes are entirely insoluble in water and polar organic solvents but soluble in halogen and alkyl-substituted benzene, CS_2, 1,2-dichlorobenzene, and naphthalene. They form stable colloid aggregates in water when treated with organic solvents and intensive sonification, or after long stirring in water [10].

Fullerenes are usually described as particles consisting of a cage of 60 or more carbon atoms. In reality, fullerene molecules crystallize into larger structures – clusters of crystals, termed nano- or colloidal fullerenes. Therefore, it is common to denote carbon molecules as carbon-clusters or nano-carbon materials. Several chemical and physical means are in use to enhance dispersion and minimize cluster/crystal size [9]. Hydrophobicity of "pristine" (unmodified) fullerene C_{60} was overcome by creating a variety of derivatives that exhibit greater water solubility. Unique structural and electronic characteristics of C_{60} are the background for diverse chemical modifications leading to a wide variety of biologically active water-soluble fullerene derivatives. By combining nucleophilic and electrophilic additions, cycloadditions, and radical additions, it is possible to covalently attach a large number of organic compounds to the fullerene cage (Figure 1).

Among water-soluble fullerene derivatives, fullerenols (fullerols) are produced by hydroxylation of fullerene molecule surface ($C_{60}(OH)_n$ 44 ≥ n ≥ 2). Our team reported on synthesis of $C_{60}(OH)_{24}$ fullerene derivative in 1997 [12].

Thus far, both non-derivatized and derivatized fullerenes showed various biological activities such as antiviral, antibacterial, antioxidative and prooxidative, neuroprotective, cytotoxic and cytoprotective, activity in cell signaling pathways, and anticancer activity [4, 10].

CARBON NANOMATERIALS IN INDUSTRY AND BIOMEDICINE

Due to their "nano" dimensions (<100 nm) fullerenes are defined as nanomaterial. Presently, fullerenes are an indispensable nanostructured material in nanotechnologies including nanomedicine primarily due to their nano size and the possibility for diverse surface modifications [13].

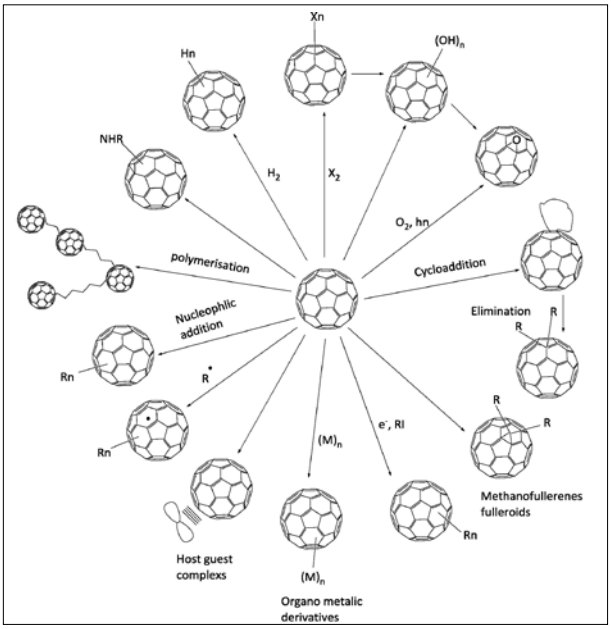
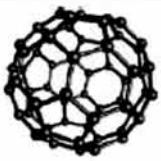
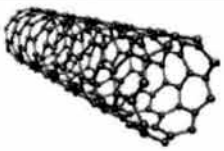
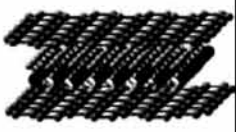


Figure 1. The chemical reactions on fullerene C_{60} (reproduced with permission from Djordjevic et al [47])

Nanotechnology is science that involves the manipulation of materials at a nanometer scale. Carbon nanotubes (CNTs) and graphene layers share remarkable properties which arise from their common structure. They are widely used in many applications (Table 1). Both CNTs and graphene have found practical applications in the same fields (flexible electronics, batteries, sensors, touch screens, fuel cells, composite materials for airplanes and automobiles). CNTs have one property that graphene sheets don't possess – they can be very long. However, the main shortcoming of CNTs is their costly production, which is difficult to control [13]. Carbon nanotubes exist in several forms. They are distinguishable by the number of walls [single-walled (SWCNTs); double-walled (DWCNTs); multi-walled (MWCNTs)], length, shape, surface modification, purity, and their propensity to form agglomerates and aggregates [14]. Metal catalysts (Co, Fe, Ni, and Mo) are involved in CNTs synthesis, which may result in the presence of residual metal contaminants. Surface modification of CNTs (by proteins, polymers, or metals and polar functional groups) may improve their dispersion in solvents or provide specific function, which influences their biological behavior. However, important properties of CNTs, such as nano-size, fiber-shape, and graphitic nature, are associated with their potential to harm human health and the environment [14, 15].

Graphene is one-atom-thick flat sheet of carbon atoms without additional functional groups. Graphene does not form stable dispersions in water or other biologically relevant solvents. It is a relatively inert and potentially non-toxic nano-material [14]. A single layer of graphene is simultaneously the thinnest, strongest, and stiffest material so far, and an excellent conductor of both heat and electricity [13]. Graphene is also easier to disperse in resin

Table 1. Opportunities and challenges in biomedical applications for different forms of nanocarbon (reproduced with permission from [28]).

	Fullerenes	Carbon nanotubes	Graphene materials	2D heterostructures
				
Unique properties used in biomedical and life science	Free radical scavenging	Cylindrical shape Photothermal capability Inner space (for filling)	2D flat shape Large available surface area Flexibility Electrical conductivity Absence of bandgap Aqueous solubility (in the case of graphene oxide) Versatility of chemical functionalization	Similar to graphene materials in shape and structure More variability in the 'mix' of physical properties from different single layers
Biomedical application (most mature or intensively explored)	Antimicrobials	Molecular transporters (drug delivery) Near-infrared imaging agents	Highly sensitive biosensors Molecular transporters Coatings/substrates for tissue engineering and implants	Almost no such applications reported Biosensing and biodetection will be prime candidates
Opportunity	Interaction with double-stranded nucleic acids (for example mitochondrial DNA)	Translocation of biological membranes and barriers	Responsive to a wide range of parameters High sensitivity Multiple read-out routes Ease and speed of degradation	'Fabricate-by-design' based on the selection of layers
Challenge	Aqueous dispersibility Non-specific DNA binding leading to cytotoxicity	Controlled manufacturing and surface functionalization Aqueous dispersibility Adverse (inflammatory) responses related to fibre shape Slow kinetics of biodegradation	Unknown cytotoxic limitations Controllable dimensions Determination of *invivo* biodegradability kinetics	Interaction with biological matter is currently unknown

than nanotubes, which makes it an important carbon nanomaterial in various nanotechnology areas. However, a common problem for the introduction of graphene in industrial applications is the lack of methods for large-scale production of graphene materials, in particular of electronic-quality layers [16].

"Nanomedicine is the manipulation of human biological systems at molecular level using nanoscale or nanostructured materials" [17]. In the past two decades nanocarbon allotropes (fullerenes, nanoparticles, nanotubes, graphene and nanodiamonds) also attracted much attention for biomedical application [18–20]. Unique physical and surface characteristics of nanoparticles such as high surface area, facile surface modification, small size, and magnetic and optical properties, make them promising candidates for biomedical applications [21].

Nano-scale materials, in general, interact effectively with biological systems. Biological response to nanomaterial depends on many factors (exposure level, systemic accumulation and excretion profiles, tissue and organ distribution, and the age of the subject), which must be considered when designing nanomaterials for clinical use with the aim to minimize nanoparticle toxicity [17].

Because of their bonding structure (classified into sp^2 and sp^3 carbon nanomaterials) nano-carbons possess fluorescent and photoacoustic emission properties that make them a useful contrast agent in optical and imaging sensing [22, 23, 24]. A variety of nanoparticles have been designed so far and many of them contain both drug and imaging agents for simultaneous disease diagnosis and therapy (theranostic nanoparticles) [17, 21, 25, 26, 27].

Fullerenes have shown potential application in photodynamic therapy, photothermal treatment, radiotherapy, and chemotherapy. They are also used as a novel contrast agent in magnetic resonance imaging (MRI) [21, 24, 26]. Besides fullerenes, graphene and its derivatives have also raised interest for biomedical application [28, 29, 30].

BIOLOGICAL ACTIVITY OF FULLERENE C_{60} AND DERIVATIVES

Fullerene C_{60} is insoluble in water and polar organic solvents but it can form relatively stable colloid aggregates (n-$C_{60,}$ 20–140 nm) when treated with organic solvents and intensive sonification, or after long stirring in water [10].

Manipulation of surface chemistry has created diverse populations of fullerenes, which exhibit different behaviors. Several approaches were included in improving water solubility of fullerenes: surface modification, solvents, extended stirring, and mechanical processes. However, some of these processes, especially the use of solvents, may influence fullerene toxicity [11].

The electron system of fullerene C_{60} makes it sensitive to visible and ultraviolet light spectrum. Numbers of *in vitro* studies showed that production of singlet oxygen underlines phototoxicity of fullerene C_{60}, and that residual quantities of different organic solvents were found responsible for the fullerene toxicity [11, 14, 15, 31].

Thus far, both non-derivatized and derivatized fullerenes showed various biological activities such as antiviral, antibacterial, antioxidative and prooxidative, neuroprotective,

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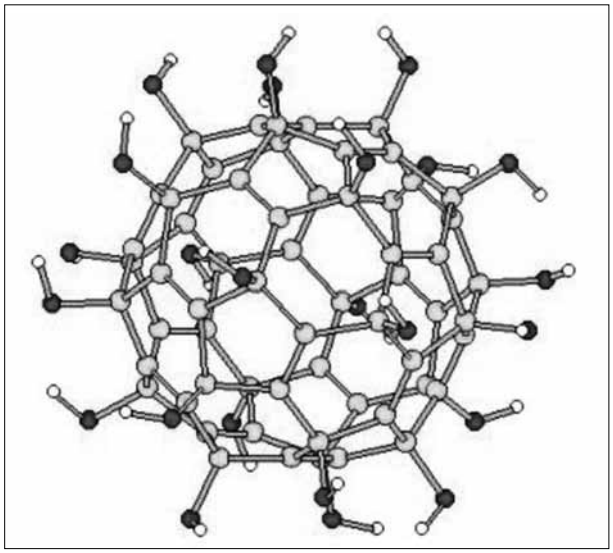

Figure 2. Water-soluble fullerene derivative, fullerenol C60(OH)24 (reproduced with permission from [4])

cytotoxic and cytoprotective, activity in cell signaling pathways, and anticancer activity. Oxidative, genotoxic and cytotoxic responses are cellular processes that underline observed fullerene toxicity. The antioxidant/cytoprotective properties of fullerenes have been considered within *in vivo* and *in vitro* experiments in the prevention of organ oxidative damage and treatment of oxidant-mediated diseases [4, 11, 32–40].

SYNTHESIS AND BIOLOGICAL ACTIVITIES OF POLYHYDROXYLATED FULLERENE C_{60} – FULLERENOLS

The chemical modification of fullerene C_{60} molecule by attachment of hydroxyl groups is an easy and straightforward method to synthesize water-soluble fullerenes, named fullerenols $C_{60}(OH)_n$. Our team synthesized fullerenol in a two-step process [12, 41]. First compound, polybromide derivative $C_{60}Br_{24}$, was obtained in catalytic reaction ($FeBr_3$) of C_{60} and Br_2. Polyhydroxylated derivative with 24 hydroxyl groups, $C_{60}(OH)_{24}$, was synthesized in alkaline media by compete substitution of bromine atoms from $C_{60}Br_{24}$ (Figure 2). Biological activity of this derivative was investigated *in vivo* and *in vitro* using various experimental model systems.

Our research team has investigated *in vitro* and *in vivo* biological activity of fullerenol $C_{60}(OH)_{24}$ for nearly two decades. Contrary to many other studies, we used fullerenol mainly at nanomolar concentrations and without photoinduction. Here, we present and discuss the results of fullerenol antioxidative and free radical scavenger activities in chemical and biological systems; cytotoxicity against human tumor cell lines; protective effect against various cytotoxic drugs and irradiation; effects on cell cycle and apoptosis, *in vitro* and *in vivo* radioprotective and cardioprotective effects; biodistribution study of radiolabeled fullerenol, and characteristics of a new doxorubicin-fullerenol nanocomposite.

ANTIPROLIFERATIVE AND CYTOTOXIC ACTIVITY OF $C_{60}OH_{24}$

Similar to non-hydroxylated water-soluble fullerene derivatives, the most important characteristics of fullerenols regarding mechanisms of their biological activity are photosensitizing property and free radical scavenging activity. These properties are important not only in medicine (e.g. disease treatment, disinfection) but also in environmental research (e.g. acceleration of oxidative decomposition of organic compounds).

Antiproliferative and cytotoxic effects of water-soluble derivatives $C_{60}(OH)_n$ have been observed in different experimental models. In several studies, fullerenols show less or negligible cytotoxicity compared to pristine C_{60} against human and animal normal and malignant cell lines [42, 43]. These studies support the opinion that water-soluble functional groups on the surface of the fullerene molecule dramatically decrease the toxicity of C_{60}. However, it has recently been shown that fullerenols may produce both singlet oxygen and superoxide under polychromatic visible and UV light [43, 44].

Our preliminary studies on antiproliferative activity of fullerenol $C_{60}(OH)_{24}$ against tumor cell lines showed that fullerenol at nanomolar concentrations had induced low and transient growth inhibition of several tumor cells. Fullerenol also induced small changes in cell cycle distribution, DNA synthesis and mitotic activity of K562 cells: mitotic index was decreased during the 72-hour incubation period up to 30%; small increase of cell number in G2M phase and the proportion of sub-G1 cells was higher in comparison to control: 11–13% vs. 3–8%. These results indicated cytostatic rather than cytotoxic fullerenol activity against K562 cell line [45].

Antiproliferative activity of fullerenol against three human breast cancer cell lines showed that fullerenol alone induced mild cell growth inhibition that was cell line-, time- and concentration-dependent [34].

Fullerenol $C_{60}(OH)_{24}$ is considered a strong free radical scavenger and potential metal ion chelator. Therefore, we tested it in a system of antitumor drug-induced cytotoxicity against different human breast cancer cell lines [46]. Doxorubicin, cisplatin, taxol, and tiazofurin were used at IC_{50} concentrations, and fullerenol at a range of nanomolar concentrations. Different schedules of fullerenol and antitumor drugs were also used. Simultaneous application of fullerenol and antitumor drugs resulted in strong suppression of antitumor drug-induced cytotoxicity. The rate of cytotoxicity inhibition depended on fullerenol concentration, type of antitumor drug and cell line. Protective effect of fullerenol was more pronounced against doxorubicin, cisplatin, and tiazofurin, drugs whose toxicity was based on reactive oxygen species (ROS) formation. Modulation of taxol-induced cytotoxicity by fullerenol might be mediated by other mechanisms, e.g. effect on cytoskeleton [34, 46].

ANTIOXIDATIVE ACTIVITY OF $C_{60}(OH)_{24}$

Antioxidative activity of fullerenol is one of its main characteristics and it is often used to explain the protective role of fullerenol in the models that generate ROS and oxidative stress [33, 40, 47].

The antioxidative activity of fullerenol $C_{60}(OH)_{24}$ was tested in chemical and biological systems.

In a chemical system, we measured fullerenol's ability to scavenge stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and reactive hydroxyl radical ($^{\cdot}OH$) during the Fenton reaction using electron spin resonance (ESR) spectroscopy [48]. The fullerenol successfully scavenged both types of free radicals. Inhibition of ESR signal of DPPH radical and the spin adduct, DMPO (5,5-dimethyl-1-pyrroline-N-oxide)–OH, was concentration-dependent. Generally, higher concentrations of fullerenol (0.71–0.88 mmol/l) inhibited the hydroxyl radical (50–82%) better than the DPPH radical (28–50%). A possible mechanism(s) of fullerenol antioxidative activity was suggested: simultaneous hydrogen atom donation to DPPH and $^{\cdot}OH$; in reaction with $^{\cdot}OH$, the generation of the fullerenol radical $C_{60}(OH)_{23}O^{\cdot}$ appeared, which was confirmed by ESR; interaction between hydroxyl radical and fullerenol are also based on a radical addition reaction of 2n $^{\cdot}OH$ radicals to remaining double bonds of fullerenol core to yield $C_{60}(OH)_{24} + 2n^{\cdot}OH$ (n = 1-12).

In vitro investigation of the possible nitric oxide-scavenging activity of $C_{60}(OH)_{24}$ showed that fullerenol expressed direct scavenging activity towards nitric oxide radical (NO) released from sodium nitroprusside (SNP) solution. *In vivo* studies of NO-scavenging activity of fullerenol were investigated on the model of testicular antioxidant enzyme activities of adult male Wistar rats treated with SNP. Pretreatment of animals with fullerenol (60 μg / each testis) prevented NO-induced decrease of catalase, glutathione transferase, and glutathione peroxidase activities in the denucleated fraction of interstitial testicular cells two hours after SNP (20 μg /each testis) treatment. The results of study showed, for the first time, direct NO-quenching activity of $C_{60}(OH)_{24}$ in different milieu. In addition, fullerenol $C_{60}(OH)_{24}$ expressed certain scavenging activity towards superoxide anion ($O_2^{\cdot-}$) and prevented lipid peroxidation [49].

Genotoxic properties of water-soluble fullerenes are still insufficiently evaluated. Contrary to phototoxic and mutagenic properties of C_{60}, fullerene derivatives including fullerenol showed no mutagenic or genotoxic effects up to milligram concentrations [11]. We investigated *in vitro* genotoxic effect of fullerenol $C_{60}(OH)_{24}$ using various cell types – Chinese hamster ovary (CHO) cell line as a standard model, human peripheral blood lymphocytes (PBL), and breast cancer cells. Fullerenol $C_{60}(OH)_{24}$ was tested in a range of concentrations depending on experimental model. Genotoxic effects were evaluated using standard sister chromatid exchanges assay (SCE), cytokinesis-block micronucleus assay (MN) and chromosome aberration assay (HA). Results of SCE test on two human breast cancer cell lines showed that fullerenol at nanomolar concentrations did not significantly affect the incidence of SCE and MN compared to the control [46]. Fullerenol (11–221 μmol/l) also reduced the frequency of MN and HA in mitomycin (MMC) treated CHO cells [38]. Results of $C_{60}(OH)_{24}$ genotoxic investigation on PBL of healthy persons indicated that fullerenol did not induce structural and numerical chromosomal aberrations. However, fullerenol (5.54–221.6 μmol/l) reduced the frequency of MN and HA in MMC treated PBL at a range of concentrations [50]. No stable chromosomal aberrations in human PBL were detected after treatment with 1–1,000 mg/ml of fullerenol; incidence of SCE and MN was significantly reduced, including decreased number of MNC with more than one MN [Jakimov D, unpublished data]. Our studies showed absence of fullerenol genotoxicity.

RADIO-PROTECTIVE AND CHEMO-PROTECTIVE ACTIVITIES OF $C_{60}(OH)_{24}$

During radiation treatment of cancer it is important to protect surrounding normal tissues and organs from damaging effects of radiation. Protective agents that preferentially protect normal tissue would allow increased radiation dose and thus improve the outcome of radiotherapy. At present, only sulfhydryl compound amifostine serves as radioprotector in clinical settings [51]. Water-soluble fullerenes as strong antioxidants and free radical scavengers have shown protective effects on cells damaged in oxidative stress [47].

We performed *in vitro* screening study to evaluate radioprotective activity of fullerenol $C_{60}(OH)_{24}$ at nano- and micromolar concentrations on the panel of human malignant hematopoietic cell lines subjected to high-dose irradiation [39, 52]. Radioprotective effect of fullerenol depended on both cell line and fullerenol concentration. Fullerenol at concentrations 1–10 nmol/ml was more effective in all cell lines, while 1 μmol/l of fullerenol in combination with X-ray (24 Gy) showed cytotoxic effect in all cell lines. Cell number of K652, Raji and HL60, and PC-MDS cells was decreased by 17%, 42%, and 50%, respectively. Cytoprotective effect of fullerenol was evident 24 hours after irradiation, and after 72 hours number of fullerenol pretreated (10 nmol/l) and irradiated (24Gy) K562 cells was similar to that of control cells. Morphological signs of cell damage were less pronounced in fullerenol treated cells. Cell area of fullerenol pretreated cells was 6% and 38% smaller compared to irradiated cells at 24 hours and 48 hours after irradiation, respectively [52]. Fullerenol also significantly modified the activity of antioxidative enzymes (AOEs) in irradiated K562 cells: activity of gamma-glutamyl transferase was significantly decreased, while superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities were increased in pretreated and irradiated cells. Thus, increased AOEs activity in fullerenol pretreated cells might prevent deleterious effects of irradiation-induced ROS. In general, protective action of fullerenol on irradiated cells is based on the ability of fullerenol to scavenge ROS and on its antioxidant activities, which makes fullerenol a potential radioprotector [39].

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In vivo radioprotective effects of fullerenol $C_{60}(OH)_{24}$ were evaluated on whole-body irradiated mice and rats [53, 54]. End points were body mass changes, survival rate, and changes in blood cell number. Fullerenol was given IP at doses of 10 mg/kg and 100 mg/kg, 30 minutes before irradiation (X-ray 6–8 Gy). Better body mass gain was obtained in mice that were given 100 mg/kg of fullerenol compared to 10 mg/kg of fullerenol. The results showed that higher fullerenol concentration prolonged mean lethal time of irradiated mice and it was more pronounced in mice irradiated with 7 Gy and 8 Gy [53].

In the second study we compared protective effects of the fullerenol (10 and 100 mg/kg IP) with amifostine (300 mg/kg IP) in irradiated rats [54]. General radioprotective efficacy (survival and body mass gain) of fullerenol and amifostine in rats irradiated with lethal dose of X-rays (8 Gy) was monitored during 30 days after irradiation. Tissue-protective effects (100 mg/kg of fullerenol and 300 mg/kg of amifostine, 30 min before irradiation), was carried out on rats irradiated with 7 Gy (a sublethal X-ray dose). The results showed that fullerenol is better than amifostine at preventing radiation-induced decrease of granulocyte and lymphocyte count, and at inhibiting histopathological changes in the spleen, small intestine and lungs, while amifostine had better radioprotective effects in the heart, liver and kidneys [54]. The studies on radioprotective effects of fullerenols demonstrated that it might be possible to make both a strategy for enhancing radiation treatment by fullerenols and to use them as remediation for adverse biological effects of X and γ irradiation.

Ionizing radiation (IR) induces ROS that mediate IR induced apoptosis. Also, ionizing radiation induces pro-apoptotic genes – expression program that inhibits cell survival. Overexpression of AOEs contributes to improving the cell redox state. Transcriptional and translational regulation of expression of these proteins plays a critical role in protecting cells from oxidative damage. The sensitivity of leukemia cells to apoptosis induced by IR is achieved by an increased production of ROS or reduced activity of AOEs. The aim was to examine whether and how fullerenol modulate the response of K562 leukemic cells to low dose irradiation. We analyzed the expression level of mRNA for 11 genes in the irradiated and fullerenol-protected K562 cells, and compared the gene expression level with the overall cell survival. Our results showed better survival of K562 cells pretreated with fullerenol (10 mmol/l) and irradiated with 2 Gy, and significant overexpression of antiapoptotic (Bcl-2 and Bcl-xL) and cytoprotective genes (GSTA4, MnSOD, NOS, CAT and HO-1). These results indicated that the fullerenol exerts cytoprotective function in K562 cells, making them more tolerant to radiotherapy [40].

We also investigated possible cardioprotective effects of fullerenol $C_{60}(OH)_{24}$ on doxorubicin-induced cardiotoxicity . Our preliminary results showed that fullerenol (50, 100, and 200 mg/kg) administered IP 30 minutes before application of a single dose of doxorubicin (8 mg/kg) prevented functional (reflex bradycardia) and morphological changes (vacuolization of cardiomyocytes) induced by doxorubicin. Further organ-protective effects of fullerenol on doxorubicin- (DOX) induced toxicity were investigated on Sprague Dawley outbred rats with chemically-induced mammary and colorectal carcinomas [35, 37, 55, 56, 57]. Pretreatment of animals with fullerenol (100 mg/kg IP, 30 minutes before DOX, 8 mg/kg IP) prevented DOX-induced oxidative stress, lipid peroxidation and misbalance of GSH/GSSG level in the rat kidney [55]. DOX-induced liver and heart toxicity were significantly reduced in rats pretreated with fullerenol, which was confirmed by macroscopic, microscopic, hematological, biochemical, as well as physiological, pharmacological, and pharmacokinetic analyses [37, 56]. Similar protective effects on the kidney and liver were obtained after oral application of fullerenol in rats treated with DOX [58]. Lung toxicity induced by DOX was also reduced in animals pretreated with fullerenol, there were no significant differences in biochemical or histopathological analyses between control and fullerenol treated animals [57].

Fullerenes and other carbon nanoparticles currently serve mostly as carriers for therapeutic agents for targeted drug delivery [59]. Doxorubicin is used in the treatment of a spectrum of solid cancers, but organ toxicity, especially cardiotoxicity, and cell resistance limit its clinical efficacy. Loading DOX into various carriers enables sustained DOX release in its active form inside the cells, assuring effective DOX concentrations for longer periods of time. Our group had recently synthesized a DOX/fullerenol complex, and we examined how the complex enters cells, how much of it is retained in the cells, as well as the effect on survival of cells treated with the complex. We used two breast cancer cell lines, MCF-7 and MDA-MB-231, for which we had previously found different susceptibility to DOX and assumed that one of the reasons for the different sensitivity to DOX could be the difference in taking DOX from the media. We found that DOX alone was successfully internalized in the cells but cellular uptake of DOX/fullerenol complex was higher in both cell types compared with free DOX. According to mean fluorescence intensity, uptake of both compounds was better in MCF-7 cells, at each time point, especially for 2-hour and 4-hour treatments. High mean fluorescent intensity of free DOX and DOX/fullerenol complex in MCF-7 cells, compared to MDA-MB-231 cells, was in accordance with higher MCF-7 sensitivity. The number of viable MCF-7 cells after a 24-hour treatment with free DOX and DOX/fullerenol complex (for DOX concentration of 0.1 μmol/l) was significantly decreased ($p < 0.01$) [Djordjevic A et al., in press].

PHARMACOKINETIC AND BIOCOMPATIBILITY STUDIES OF FULLERENOL

To assess activity of water-soluble fullerenes as potential drugs it is necessary to explore their pharmacological behavior and metabolism. Radiolabeled fullerenes are needed in order to obtain data on their absorption, distribution and excretion [60]. Pharmacokinetic and biocompatibility studies of fullerenes are still limited [11, 37]. As exposure

to fullerenes could occur via different routes (oral, dermal, pulmonary, or injection route) the toxicity at a site of exposure is of particular interest [11].

Biodistribution of newly synthesized radiopharmaceutical $^{99m}Tc(CO)_3(H_2O)_3]^+C_{60}(OH)_{24}$ was investigated on a domestic dog during 24 hours using dynamic and static scintigraphy. Thirty-minute dynamic scintigraphy detected the radiopharmaceutical in the heart, liver, and spleen. Activity in the heart rapidly declined, while the activity in the liver and spleen increased in the first 10 minutes and subsequently stabilized for 30 minutes. By static scintigraphy, one hour after administering, γ activity was detected in the heart, liver, spleen, and intestine, and in salivary glands and intestines after four hours. Twenty-one hours after administering, the radiopharmaceutical was detected in the spleen, liver, salivary glands, kidneys, and urinary tract, but not in the small intestine; however, after 24 hours, γ activity was detected only in the liver, spleen, and urinary tract. Radiopharmaceutical $^{99m}Tc(CO)_3(H_2O)_3]^+C_{60}(OH)_{24}$ is eliminated via urinary tract [60, 61].

Studies on biodistribution of fullerenols published so far indicate similar clearance pathways, tissue retention, and tissue distribution. That makes $C_{60}(OH)_x$ and other fullerenol-like materials potential therapeutic agents for treating leukemia, bone cancer, or bone pain. They also might serve as photosensitizer in the photodynamic therapy of some kinds of tumor [62].

THERANOSTIC NANOPARTICLES AND POTENTIAL CANCER STEM CELLS THERAPEUTICS

Over recent years, there has been a trend of developing nanoparticle constructs with more than one functionality. A number of such constructs named "theranostic nanoparticles" are able to simultaneously target, enable real-time imaging, and deliver drugs. The ultimate goal of theranostic medicine is more effective and personalized treatment approach. Thus far, various theranostic nanoparticles were produced combining therapeutic strategies (nucleic acid delivery, chemotherapy, hyperthermia, and photodynamic and radiation therapy) with one or more imaging functionalities. Besides gold and iron oxide nanoparticles as nanoplatforms, nano-carbon allotropes (fullerenes, carbon nanoparticles and nanotubes, graphene and nanodiamonds) have attracted interest as nanostructures for biomedical application [17, 18, 21, 26, 27, 63]. Recently, metallofullerenol $[Gd@C_{82}(OH)_{22}]_n$ was developed and tested *in vivo* as theranostic medicine [17]. Originally designed as a contrast agent for MRI, it showed 12 × MRI relaxivity compared to commercial Gd-DTPA. Treatment of mice bearing hepatoma and breast cancer with $[Gd@C_{82}(OH)_{22}]_n$ nanoparticles resulted in tumor growth inhibition. Tumor inhibition was achieved by improving the immune processes (stimulation of T-cells and macrophages) and by decreasing tumor microvessel density, thus lowering the speed of blood supply to tumor stroma without effecting normal capillary vessels. Coadministration of nanoparticles with cisplatin activated endocytosis in the human cisplatin-resistant cancer cells, which resulted with intracellular drug concentration increase and formation of cisplatin-DNA adducts. $[Gd@C_{82}(OH)_{22}]_n$ protected normal cells by scavenging ROS and inhibiting lipid peroxidation *in vivo*, thus contributing to low toxicity of theranostic nanoparticles. A toxicological study proved that there were no clear changes between controls and animals treated with nanoparticles at therapeutic doses [17].

Liu et al. [64] have recently studied the report on Gd-metallofullerenol intrinsic anticancer properties. Gd-metallofullerenol, $Gd@C_{82}(OH)_{22}$, was nontoxic to normal mammary epithelial cells, but showed intrinsic inhibitory activity against triple negative breast cancer cells by blocking epithelial-to-mesenchymal transition (EMT) and depleting breast cancer stem cells (CSC). The activity resulted in abrogation of both tumor initiation and metastasis. The mechanism by which $Gd@C_{82}(OH)_{22}$ blocks EMT and reduces CSC population in breast cancer cell lines was mediated by TGF-beta signaling and HIF-1-alpha activities. Gd-metallofullerenol has been for the first time identified as a specific CSC inhibitor. Another carbon nanostructure, graphene oxide, a water-soluble derivative of graphene, showed selective inhibition of the proliferation expansion of cancer stem cells of multiple tumor types. Graphene oxide was nontoxic for the tumor non-stem cells and normal fibroblasts and effects to stem cells were exerted by inducing stem cell differentiation through inhibition of key signal transduction pathways – WNT-, Notch-, and STAT-signaling [30].

FULLERENE TOXICITY

To date, little is known about carbon nano-clusters' potential to harm both human health and the environment [26]. Non-biodegradability and toxicity of carbon nanoparticles still remains a great concern in the area of biomedical application [25]. It should be pointed out that the same physiochemical properties of nanomaterials might also be associated with potential health hazards [65].

Exposure to fullerenes could occur via different routes. Therefore, toxicity at the site of exposure is of particular interest. So far, a limited number of studies address ADME (adsorption, distribution, metabolism and excretion) profile of fullerenes [11]. Available studies suggest that the majority of fullerenes remain at the deposition site, although some can cross cell barriers and be transported within the blood. Fullerenes are predominantly accumulated in the places of their toxicity – the liver, kidneys, and spleen (4, 11). It is also suggested that metabolism of fullerenes may occur in the liver, and elimination of fullerenes occurs within urine and feces, which may reduce their propensity for distribution and toxicity.

Distribution study of fullerenes following IV injection is important primarily due to their potential use as drug carriers. Different studies show that, subsequently, the injected (either IV or IP) fullerenes are accumulated in the liver. This finding is relevant with respect to several topics: im-

pact of fullerene accumulation on liver functions, fullerene metabolism in the liver, and elimination route of fullerenes by bile and feces (for more information see [4] and [11]).

In vitro studies of dermal and cardiac toxicity of fullerenes showed that fullerene type, skin condition, and experimental protocol may influence inflammogenic and cytotoxic potential of fullerenes to the skin, while conflicting results were reported regarding prothrombogenic potential of fullerenes. Ocular toxicity of fullerenols was enhanced by UVA and visible light exposure, which confirmed photosensitive aspect of fullerenols.

According to the studies conducted so far, fullerene toxicity is mediated by oxidant-driven response: oxidative stress and its consequences, such as inflammation and genotoxicity. Toxicity studies of fullerenes are limited in regard to experimental model, investigated targets, and mechanisms of toxicity. Therefore, generalization about fullerenes behavior and toxicity is not possible thus far [11].

CONCLUSION

The unique physical and surface features of nanoparticles made them promising candidates for biomedical application. However, size, shape, and composition of nanomaterials may all be attributed to or associated with adverse health effects.

Carbon nanoparticles attracted much attention for biomedical application in the past two decades. Fullerenes have showed potential for application in different cancer therapeutic approaches. In addition, they can also be used as novel contrast agents in MRI. Apart from fullerenes, graphene has appeared as an interesting nanomaterial for biomedical application. Although great advances have been achieved in the past two decades using carbon nanomaterials in therapy and diagnostics of diseases, their application may be associated with potential health hazards. Non-biodegradability and toxicity of carbon nanoparticles still remain a serious concern regarding human health and environmental hazards. Therefore, further studies are required to explore such potential effects.

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Угљенични наноматеријали: биолошки активни деривати фулерена

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КРАТАК САДРЖАЈ

Фулерени, угљеничне наноцеви и графен су од самог открића значајно привлачили пажњу истраживача у разним научним областима укључујући и биомедицину. Угљенични наноалотропи су захваљујући својим нанодимензијама и разноврсним модификација на њиховој површини постали незамењив наноматеријал у нанотехнологијама укључујући и наномедицину. Хемијским модификацијама на површини фулерена створене су разнолике групе водорастворних деривата фулерена, који се различито понашају у биолошким системима. И дериватизивани и недериватизовани фулерени испољили су различите биолошке активности. Токсичност фулерена почива на три ћелијска процеса: оксидативном, генотоксичном и цитотоксичном одговору ћелије. Антиоксидативна/цитопротективна својства фулерена и њихових деривата испитивана су у превенцији и у лечењу оштећења органа изазваних оксидативним стресом. Сва јединствена физичкохемијска својства наноматеријала могу бити и потенцијално штетна по здравље. Биолошка неразградљивост и токсичност угљеничних наночестица и даље представљају главни проблем за њихову примену у биомедицини. У овом прегледном раду приказана су основна физичка и хемијска својства угљеничних нанокластера – фулерена, наноцеви и графена, њихове специфичности, активности и потенцијална примена у биолошким системима. Посебно су истакнути најважнији резултати добијени *in vitro* и *in vivo* са полихидроксилованим дериватом фулерена $C_{60}(OH)_{24}$.

Кључне речи: фулерен C_{60}; фулеренол; наночестице; угљенични наноалотропи; хватачи слободних радикала; ћелијске линије; цитотоксичност; антитуморски лекови

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