# Fullerenes in Biology and Medicine

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Abstract

Fullerenes and related carbon based derivatives have shown a growing relevance in biology and medicine, mainly due to the unique electronic and structural properties that make them excellent candidates for multiple functionalization. This review focuses on the most recent developments of fullerene derivatives for different biological applications.

Graphical abstract
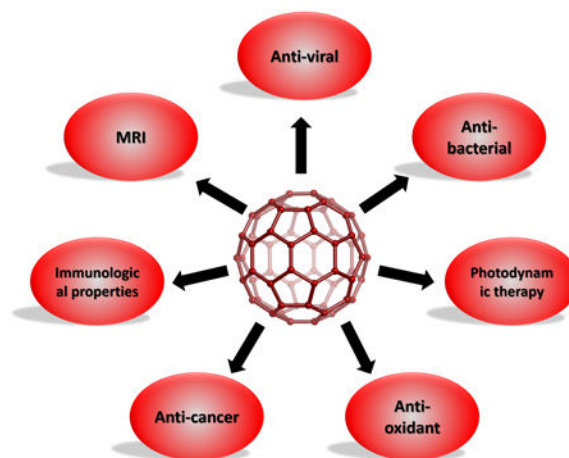
1. Introduction

The research on fullerene C_{60} started in 1985 when Kroto, Curl and Smalley discovered the third allotropic form of carbon after diamond and graphite.[1] The first functionalization reaction[2] was reported soon after the fullerenes were synthesized in macroscopic amounts in 1990.[3] Functionalized fullerenes exhibit improved solubility and different chemical and physical properties that allow applications in multiple fields.[4] In 1993 Wudl and coworkers reported the first water-soluble fullerene derivative **1** (Fig. 1) that inhibited the activity of the HIV-1 Protease (HIV-1 PR), as determined from the interaction of this compound and the HIV-1 PR on an in vitro assay.[5] Since then, efficient synthetic methods for fullerene functionalization have been developed.[6, 7] Functionalization with bioactive compounds that exhibit affinity to certain nucleic acids, proteins or cell receptors such as peptides[8-10] or

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saccharides,[11, 12] functionalization with polar or ionic groups such as hydroxyl,[13, 14] carbonyl[15-17] or quaternary ammonium salts,[18, 19] and encapsulation with macromolecules[20, 21] are the most common approaches to synthesize water-soluble fullerene derivatives for biomedical applications.[22-26]

Several reviews have been published that summarize the biological applications of fullerene derivatives.[23, 27, 28] In this review, we summarize the biological properties of active fullerene derivatives reported during the last 3 years along, with their biomedical applications.

2. Properties

The three dimensional shape of fullerenes results in unique chemical and physical properties. Chemical functionalization can be easily achieved to alter the properties of the new compounds.[7, 29-31]

2.1 Chemical properties

Due to the spherical shape of fullerenes, carbon atoms are highly pyramidalized and are thus highly reactive. The conjugated carbon atoms deviate from planarity by orbital rehybridization of the sp^2 orbitals to $sp^{2.27}$ orbitals with a p-character gain.[32]

C_{60} and C_{70} fullerenes can be reversibly reduced with up to six electrons.[33] This high electron affinity results from the presence of triply degenerate low-lying lowest unoccupied molecular orbitals (LUMOs).[34]

The chemical reactivity of fullerenes is similar to that of an electron deficient olefin, yielding cycloaddition reaction products[35] where the addition takes place at a 6,6 ring junction. Additions to 5,6 ring junctions have been reported as a result of rearrangements after the initial kinetic addition to a 6,6 bond.[32]

2.2 Physical properties

Fullerenes can resist high pressures and return to their original shape after being subjected to over 3000 atmospheres.[36] It was theoretically calculated that a single C_{60} molecule has an effective bulk modulus of 668 GPa when compressed to 75% of its size. This property makes fullerenes harder than steel and diamond, whose bulk moduli are 160 GPa and 442 GPa, respectively.[37]

Fullerenes are the only known allotropes of carbon that can be dissolved in some common solvents at room temperature. Large indices of refraction, a dielectric constant of approximately 4, large molecular volumes, and Hildebrand solubility parameter equal to 10 $cal^{1/2}cm^{-3/2}$ have been reported for C_{60} solutions.[38]

3. Antiviral activity

3.1 Anti HIV-1

In the absence of an effective vaccine, antiretroviral therapy (ART) is the only therapeutic tool that can be used to treat HIV-1 infection. Usually, this protocol combines a nucleoside

reverse-transcriptase inhibitor (RTI) with either a non-nucleoside RTI or a protease inhibitor (PI).[39] These ARTs efficiently suppress the spread of HIV in patients; however, the emergence of multidrug resistant viruses is still a serious problem in anti-HIV therapy.[39-41] Therefore, the development of new type of anti-HIV drugs that are structurally distinct from the currently used pharmaceutical agents is a critical need.

It has been demonstrated in vivo and in vitro that impairment of viral maturation is a powerful strategy to block HIV-1 replication. HIV-1 maturation can be inhibited by PIs or by maturation inhibitors (MIs). The latter can bind to Gag and affect its processing or to CA, thus impairing core assembly.[42] Currently, there are no maturation inhibitors used clinically.[43]

HIV PIs work by specifically binding to the active site and blocking the activity of the protease. For 24 years, it was assumed that fullerene inhibition of HIV-1 was only due to the interaction between the fullerene and the hydrophobic pocket of this enzyme, as determined by *in silico* predictions combined with an *in vitro* assay.[5, 44] This paradigm was recently challenged by the fact that C_{60} and C_{70} fullerene derivatives do not inhibit the activity of HIV-1 PR at concentrations (3 μM) that potently inhibit HIV-1 infection (see structures in Fig. 2).[19, 45] Additionally, fullerenes **2**-**6** inhibited HIV-1 viruses resistant to multiple PR and MIs. Although the mechanism of action is not known, it was suggested to be via impairment of virus maturation as a result of a strong interaction with the immature capsid in pull-down experiments.[19] Fullerene derivatives **2**-**6** were reported to exhibit no cytotoxicity, therefore they are excellent candidates as anti HIV-1 agents.[19, 45] Due to the solubility of [60]fullerene-peptide derivatives, they have also been considered as potential inhibitors of HIV-1 replication.[46] Using drug screening and docking calculations, a database of PI [60]fullerene derivatives and their binding constants were predicted.[47] Strom *et al.*[48] determined that improved inhibition can be achieved with minor modifications of both the peptide and the amino acid structure.

PR inhibition by [60]fullerene peptides **7**-**11** (Fig. 3) was predicted *in silico*, and binding constants were calculated experimentally using a cell-free fluorescence resonance electron transfer (FRET) assay.[48] Recently Khakina *et al.*[49] reported a new fullerene derivative **12** (Fig. 3) that exhibits low cytotoxicity (above 100 μM) and blocks the HIV-1 infection in vitro, with a minimum inhibitory concentration (EC_{50}) of 4.6 μM. Compound **12** showed higher anti HIV-1 activity than its pentasulfide analogue $C_{60}(SC_2H_4COOK)_5H$ ($EC_{50} = 45.2$ μM).[50] However, no mechanism of action was proposed for this antiviral compound.

As previously mentioned, the HIV-1 reverse transcriptase (RT) is required for early proviral DNA synthesis and thus inhibition of this enzyme results in inhibition of the virus replication. The HIV-1 RT inhibitory activity of cationic, anionic and zwitterion fullerene derivatives have been previously reported,[51] where cationic fullerenes showed lower activity. Yasuno *et al.*[52] reported for first time that pyridine/pyridinium-type fullerene derivatives **13**-**15** (Fig. 3) without any carboxylic acid, inhibit the HIV-1 RT in a cell-free environment, with no cytotoxicity and clearly showing that the fullerene cage was crucial for the inhibition of HIV-RT. Compounds **16**-**24** (Fig. 3), were also active against HIV-RT.

3.2 Bacterial and viral inhibition via anti-adhesion to host cells

3.2.1 Anti-bacterial inhibition—Molecular recognition between carbohydrates and proteins play and essential role in many biological processes.[53] One example is bacterial infection that begins with bacterial adhesion to host cells. Inhibition of this step using glycoclusters or glycodendrimers that mimic multivalent carbohydrates at the cell surface is one of the most studied mechanisms over the last decade.[53] Protein-carbohydrate interactions exhibit high specificity and weak affinities toward carbohydrate ligands. It has been demonstrated that ligand multivalency increases the low binding affinities of monovalent glycosides.[53]

Nierengarten et al.[54] have demonstrated that fullerenes are very attractive spherical scaffolds that can be functionalized with carbohydrates using a very straightforward approach. Using this synthetic approach Sánchez-Navarro et al.[55] reported new hexakis adducts of C_{60} endowed with 24 sugars moieties. The carbohydrate recognition of these new derivatives by a lectin was investigated by isothermal titration calorimetry (ITC) experiments. The results showed that fullerene C_{60} is an efficient platform for the globular presentation of carbohydrates for protein-carbohydrate interactions. Buffet et al.[56] reported the synthesis and the multivalent effect against two lectins, LecB and RSL (compounds **25A**-**25E**) (Fig. 4). The results showed higher affinity for compound **25E**, which contains higher valency and a longer linker between the core and the sugar. This fullerene derivative appears to be one of the best inhibitors for both lectins.

3.2.2 Anti Ebola-virus—Ebola virus infection causes severe and often fatal hemorrhagic fever in humans and other mammals. The 2013-2015 Ebola virus epidemic in West Africa[57] caused at least 11,310 confirmed deaths.[58] The virus initiates infection by binding to host receptors through its glycoprotein surface. DC-SIGN, a C-type lectin receptor has been proposed as one of the receptors for cell entrance for several microorganisms that contain glycoconjugates on their envelope.

Due to the important role of DC-SIGN in the first step of the viral infections such as HIV and Ebola,[59, 60] the discovery of new compounds with higher affinity for this receptor is of great importance.

The sugar residues in fullerenes act as solubilizing groups and induce remarkable biological properties.[56, 61-64] In 2013, Luczkowiak et al.[65] reported for the first time the potential application of glycodendrofullerenes as antiviral agents. In 2016 Muñoz et al.[12] reported the synthesis of hexakis adducts of fullerene C_{60}, with the most effective dendrimeric growth (compounds **26**-**28**) (Fig. 5). Compounds **25** and **27** showed very potent antiviral activity in the range of nM and pM concentrations, respectively. The results clearly showed the dependence of the inhibition effect on mannoses, compound **26** with 120 galactoses was not active.[12]

4. Photodynamic therapy

Photodynamic therapy (PDT) is a clinically approved therapeutic technique for the treatment of multiple diseases, mainly cancer.[66] It relies on the photoexcitation of an inactive

photosensitizer (PS) at a wavelength that matches the absorption of the PS, leading to localized generation of cytotoxic reactive oxygen species (ROS). ROS cause oxidative damage to nearly all types of biomolecules (proteins, lipids and nucleic acids) and tumor cell death.[67] PDT has remarkably improved selectivity and fewer side effects than traditional anticancer therapies.[68]

Fullerenes C_{60} and C_{70} can effectively be excited to their triplet state and can efficiently convert $^3O_2$ to $^1O_2$.[69, 70] Hamblin and coworkers[71-74] have tested electron-transfer reactions of photoexcited cationic fullerene derivatives both in vitro and in vivo on animal models with localized bacterial infections. The activity of compound **29** (Fig. 6) was significantly improved by the addition of potassium iodide (KI) salt, although how the iodide anion enhances the antibacterial effects is not completely understood.[72]

Functionalization of C_{60} with *n*-butyl sulfonic acid salts to form self-assembled molecular micellar nanospheres, as with compound **30** (Fig. 6), was found to retain high efficiency in generating singlet oxygen upon irradiation with visible light.[75] The mechanism was correlated to the facile intermolecular triplet energy-transfer from photoexcited ^{3}**30*** to molecular oxygen. Guan *et al.*[76] reported nanovesicles of fullerene C_{70} *tris*-adducts conjugated with chlorin e6 (Ce6), compounds **31**, (Fig. 6) through an oligo ethylene glycol linker. This system significantly enhanced both cellular uptake efficiency and PDT *in vitro* and *in vivo*. Biocompatibility and total body release of these particles are among the advantages of these conjugated nanovesicles.[76]

The production of ROS in mitochondria plays a critical role in photodynamic therapy. Li *et al.*[77] investigated the generation of additional endogenous ROS in mitochondria using a chitosan oligosaccharide grafted fullerene conjugate (CS–C_{60}) compound **32** (Fig.6). The ROS generated at low doses of CS-C_{60} was rich in mitochondria, enhancing the inhibition of human malignant melanoma (A375) cells. Rotenone Assays showed that PDT-induced endogenous ROS was generated through an electron transport chain (ETC).[77] Kim *et al.*[78] reported the synthesis of chitosan/fullerene nanogel compound **33** (Fig. 6) for efficient tumor therapy. At pH 7.4, electrostatic interactions between the pendant carboxylic acid groups of 2,3-dimethylmaleic acid (DMA) and the residual free amine groups of glycol chitosan (GC) resulted in the formation of multinanogel aggregates. Nevertheless, dissociation of the nanogel aggregates was observed at pH 5.0, resulting in improved singlet oxygen generation and elevated photodynamic tumor cell ablation.[78]

Franskevych *et al.*[79] reported the cytotoxic effect of C_{60} in combination with visible light irradiation on leukemic L1210 cell lines, both sensitive and resistant to cisplatin. Their results suggest that C_{60}-mediated photodynamic treatment is a good candidate for restoring drug-resistant leukemic cells induced to mitochondrial apoptosis. C_{60}-RITC compound **34** (Fig. 6) was synthesized to monitor C_{60} entry into leukemic cells via confocal microscopy.[79]

Mion *et al.*[80] reported the synthesis of fullerene compound **35** (Fig. 6) for photodynamic therapy and imaging applications. Although it was not completely isolated, its presence was confirmed by MALDI-TOF. Ershova *et al.*[81] studied the cytotoxicity and genotoxicity of a C_{60} fullerene derivative functionalized with five residues of 3-phenylpropionic acid and a

chlorine atom arranged around one cyclopentadienyl unit, compound **36** (Fig. 7), in diploid human embryonic lung fibroblasts (HELFs). It was found to cause complex time-dependent changes in ROS levels. At short exposure times, when it had not yet penetrated the cytoplasm, compound **36** considerably decreased ROS levels in the medium and on the surface of the cell membrane. Long-term exposure can induce secondary oxidative stress and possibly induce pulmonary fibrosis.

Fullerene C_{60} derivatives **37-39** (Fig. 7) were incorporated into liposomes using a fullerene exchange method involving the transfer of fullerenes from the cavity of two γ-cyclodextrin molecules to a liposome, as illustrated in Fig. 8.[20, 21]

The photodynamic activity of compound **38** incorporated into lipid membranes was much higher than that of compounds **37** and **39** under the same conditions. The high photodynamic activity of compound **38** was attributed to its increased $^1O_2$ generating ability, as determined by 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) as a detector.[82]

Wang *et al.*[83] reported the synthesis and DNA cleavage activity of a β-cyclodextrin fullerene C_{60}, compound **40** (Fig. 7). The DNA cleaving ability was tested by using pBR322 plasmid DNA containing more than 90% supercoiled DNA, NADH and compound **40**. DNA cleavage was observed after 4 h of visible light irradiation, but it was not observed under no light in the presence or absence of both NADH and compound **40**. Additionally, compound **40** showed no significant cytotoxicity in human neuroblastoma SH-SY5Y cells, as determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[83]

5. Antioxidant properties of fullerenol derivatives

Functionalization of fullerenes with polar functional groups is one of the approaches to overcome their insolubility in water, while retaining their unique properties and to enhance their biological activities.[84-86] fullerenes are commonly classified as a "radical sponges",[87] due to their remarkable reactivity with free radicals.[88-97]

The antioxidant capacity of Fullerenols and their derivatives have been measured by DMPO-spin trap/ESR methods, and by using the β-carotene and DPPH assays.[98] However, the kinetics and radical scavenging mechanisms of these compounds are still not completely understood. Density functional theory (DFT) calculations suggest that fullerenols with 6 and 12 hydroxyl groups are the most stable derivatives.[99]

Ueno *et al.*[100] studied the antioxidant activity of several hydroxylated fullerenols with 8, 10, 12, 24, 26, 36, and 44 hydroxyl groups by a β-carotene bleaching assay. The relative radical scavenging rate of fullerenols toward radical species derived from linoleic acid under autoxidation conditions indicated that $C_{60}(OH)_{12}$ and $C_{60}(OH)_{44}$ reacted 1.62 and 1.54 times faster than β-carotene, respectively.

Based on the analysis of the possible products of both fullerenols and C_{60} with radical species generated from methyl linoleate under autoxidation conditions, the authors proposed two antioxidant mechanisms dependent on the number of hydroxyl groups (Fig. 9).[100]

The C-addition, which includes the peroxyl radical addition to a conjugated C=C double bond (mechanism A), and the H-abstraction that involves hydrogen atom abstraction from –OH groups and the subsequent of the fullerene radical rearrangement to form ether bridge (mechanism B). Fullerenol $C_{60}(OH)_{10}$ with more π-conjugated double bonds undergoes the C-addition mechanism similar to C_{60}, while fullerenol $C_{60}(OH)_{40}$ undergoes H-abstraction because of the less π-conjugated double bonds.

Grebowski et al.[101] studied the rate constants of highly hydroxylated fullerene $C_{60}(OH)_{36}$ interacting with hydroxyl radicals (•OH) and hydrated electrons. The rate constant for the reaction of $C_{60}(OH)_{36}$ with •OH was 2.0×10^9 dm^{3}mol^{-1}s^{-1}, which was similar to that for $C_{60}(OH)_{18}$, 4.5×10^{-8} dm^{3}mol^{-1}s^{-1}.[102] These results showed that the decreased number of π bonds, due to the larger number of OH groups attached to the fullerene, did not significantly affect the rate constant for the reaction with hydroxyl radicals.

Srdjenovic et al.[103] studied the influence of $C_{60}(OH)_{24}$ on Chinese hamster ovary cells (CHO-K1). Their results showed strong antioxidant properties influencing the cellular redox state and enzyme activities. Therefore, they reduce cell proliferation and were proposed as cytoprotective agents.

Improved free radical scavenger properties were reported by Awan et al.[104] as a result of functionalization of $C_{60}(OH)_{30}$ on the surface of cellulose nanocrystals (CNCs). The immobilization appears to be due to the covalent interaction between the -OH groups on the CNC and π-conjugation of the fullerenol. It was proposed that this results in a reduced tendency to form clusters and greater accessibility to the free radicals (Fig. 10).

Mitochondrial dysfunction is considered a crucial mechanism of nanomaterial toxicity. Yang et al.[105] investigated the effects of $C_{60}(OH)_{44}$ on isolated mitochondria. The exposure of mitochondria to fullerenol not only affected the respiratory chain but also damaged the inner membrane, allowing permeability to H^+ and K^+ in a concentration dependent manner.

6. Anti cancer properties

Recent studies illustrate the wide range of avenues of the use of fullerenes as anticancer agents.[106-113] Pyrrolidinium fullerene derivatives have been shown to induce mitochondrion- and caspase-dependent apoptosis through the generation of ROS in human promyeloleukaemia (HL-60) cells.[114]

It has also been reported that pyrrolidinium fullerene compounds **41-47** (Fig. 11) induced apoptotic cell death of cells mutated on JAK2 V617F, which show resistance to multiple anti tumor drugs.[115]

The results showed that replacement of one hydrogen atom (compound **42**) or the *N*-methyl group (compound **45**) on compound **41** with a butyl group enhanced the ability of pyrrolidinium fullerenes to induce apoptosis on VF-Ba/F3 cells.[115]

Attachment of structural conjugate drugs to C_{60} is a good strategy to confer biological activity to fullerenes.[116] Magoulas et al.[117] studied the properties of fullerene C_{60}

conjugated with one (**48**) and two (**49**) units of doxorubicin (DOX), a potent anticancer agent (Fig. 11). To avoid *bis*-adduct mixture of isomers, the authors opted for multiple arms on a *mono*-fulleropyrrolidine derivative (Fig. 11). These conjugates exhibited comparable, but with a more delayed onset, antiproliferative effect compared to free (non-conjugated) DOX when incubated with MCF-7 cells under the same conditions.

Probably, after conjugate entrance into the cells, the drug must be released from the conjugate to become active.

The effect of sonodynamically activated pyrrolidine *tris*-acid fullerene derivative **50** (Fig. 11) in isolated sarcoma-180 cells, and mice bearing ectopically-implanted colon 26 carcinoma was studied by Yumiko *et al.*[118] The results showed a 5-fold enhanced cell damage induced by ultrasonic exposure in the presence of compound **50** (80 μM) and the growth of the implanted colon 26 carcinoma was decreased both *in vitro* and *in vivo* experiments. Histidine, a singlet oxygen and hydroxyl radical scavenger was used to determine whether reactive oxygen species participate in sonodynamically inducing cell damage. The results suggest that the enhancement was due to the generation of active oxygen. This work is the first step for clinical applications of sonodynamic therapy using fullerene derivatives.[118]

7. Immunological properties

It has been reported that C_{60} fullerene derivatives, when conjugated to bovine thyroglobulin or rabbit serum albumin, induce the production of fullerene-specific IgG antibodies in immunized mice.[119, 120, 121, 122] Turabekova *et al.*[123] reported a computational study where C_{60} fullerenes can act as ligands for toll-like receptors and therefore can be recognized as pathogens, inducing proinflammatory immune response.

The potential suppressive effects of compound **50** (Fig. 11) and fullerenol ($C_{60}(OH)_{36}$) was studied on many *in vitro* and *in vivo* models of inflammatory diseases by Hirai *et al.*[124] Their results suggest that both fullerene derivatives can suppress acquired immune responses that require T cells *in vitro*. Additionally, compound **50** suppressed ovalbumin-specific antibody production and ovalbumin specific T cell responses in vivo.

Funakoshi-Tago *et at.*[125] demonstrated that *bis*-malonic acid fullerene derivative compound **51** (Fig. 12) suppressed the interleukin 33 (IL-33) induced expression of IL-6 in bone marrow-derived mast cells (BMMC). IL-33 works both intra- and extracellularly as a nuclear factor regulating gene transcription and as a traditional cytokine, respectively.[126] Intracellular IL-33 is thought to be released after cell necrosis to alert the immune system of tissue damage or stress, and extracellular IL-33 coordinates immune defence and repair mechanisms while also initiating differentiation of helper T cells as the adaptive immune response is triggered.[127] This has attracted attention as a new target for the treatment of inflammatory diseases.[128]

Pacor *et al.*[129] studied the interaction between fullerene derivatives **52** and **53** (Fig. 12) and cells of the immune system and their results stress the important role of fullerene derivatives in the control of inflammatory and cancer diseases.

52 showed biological properties that make it compatible with monocytes and macrophages, remarking its potential use to deliver compounds capable of modulating the immune responses or as a vehicle to deliver anticancer drugs.[129] Compounds **52** and **53** were more active on neoplastic proliferating cells than on circulating monocytes. Compound **53** is more toxic than compound **52** against the tested cells. At active doses on tumor cells, **53** was found in the cell cytoplasm without altering the cell membrane permeability. It was not clear why **53** was toxic to macrophages, but it can be attributed to the high concentration to which the cells were exposed rather than a negative effect on mitochondrial function.

8. Biological applications of endohedral metallofullerenes (EMFs)

EMFs are fullerenes that encapsulate metal atoms or clusters. Some of the encapsulated species can be used in diagnostic or tumor therapy, but these properties are limited due to their high toxicity. It is well known that the carbon cages of EMFs prevent the encapsulated species from being released to or reacting with biological systems. Additionally, they retain enough reactivity for exohedral-functionalization that transforms the hydrophobic cage into more hydrophilic compounds as illustrated in Fig. 13.[130] The recent progress with gadolinium (Gd) based EMFs for biomedical applications is described.

8.1 Magnetic Resonance Imaging (MRI) Contrast Agents

Gd-based metallofullerenes (Gd-MF) have attracted special interest as MRI contrast agents due to their high paramagnetic properties and high relaxivity.[131-134]

Murphy et al.[135] reported the application of $Gd_3N@C_{80}$ for in vitro and in vivo imaging and tracking of human amniotic fluid-derived stem cells within lung tissue. $Gd_3N@C_{80}$ was able to specifically bind to damaged lung tissue, although the imaging intensity dissipated a month after the initial dosage. This is the first reported use of a Gd-EMF to label and deliver exogenous cells to tissue and perform longitudinal MRI to evaluate the dynamics of cell homing, migration and clearance.

Zhang et al.[136] reported a hydroxylated $Gd_3N@C_s$-C_{84}, an "egg-shaped" EMF with fused pentagons on its cage that increase the dipole moment of the compound, and thus its solubility in water. Moreover, it exhibited enhanced relaxivity. Meanwhile, Li et al.[137] reported two hydroxylated derivatives, $Gd@C_{82}(O)_{10}(OH)_{16}$ and $Gd@C_{82}(O)_{10}(OH)_{22}$, that exhibited similar properties as those of $Gd_3N@C_s$-C_{84}.

Xiao et al.[138] synthetized a new Gd endohedral fullerene derivative, IL-13-TAMRA-$Gd_3N@C_{80}(OH)_{30}$-$(CH_2CH_2COOH)_{20}$, functionalized with a cytokine IL-3 that increases its selectivity for IL-13Rα2 receptors. The former are important markers for Chronic Post-Traumatic Osteomyelitis, an opportunistic infection of the bone marrow. Gd-EMFs functionalized with the same IL-13 cytokine and additional positively charged amino groups (NH_3^+) have also been used in imaging Glioblastoma tumor cells as represented in Fig. 14.[139] The positively charged groups, coupled with the IL-3 functionalization, increased selectivity for tumor U-251 cells. Li et al.[140] studied the improved relaxivity of Gd-EMF derivatives deposited on graphene oxide nanosheets (GO-Gd@C_{82}). Their results showed that physicochemical properties and structure are important factors that can affect relaxivity.

Additionally, electron transfer between Gd-EMF and the GO sheet was found to aid proton relaxation. This interaction is stronger than that of water and the Gd ions.

Pan *et al.*[141] investigated the mechanism of Gd@C$_{82}$(OH)$_{22}$ on epigenetic regulation of human pancreatic cancer metastasis. From computational and experimental results both *in vitro* and *in vivo*, it was concluded that Gd@C$_{82}$(OH)$_{22}$ inhibits histone deacetylase-1 (HDAC-1) and suppresses pancreatic cancer cell invasion and metastasis.

Tang et al.[142] investigated the activity of Gd@C$_{82}$(OH)$_{22}$ in cancer immunotherapy by regulating macrophage activity. The results showed enhanced macrophage viability, phagocytosis, and secretion of cytokines by macrophages. The activated macrophages inhibited tumor formation and metastasis *in vivo*, and induced cancer cell death *in vitro*, as represented in Fig. 15.

The ability of Gd@C$_{82}$(OH)$_{22}$ on activating macrophages was also studied by Chen *et al.*[143] using synchrotron-based scanning transmission X-ray microscopy (STXM) with high spatial resolution of 30 nm. Gd@C$_{82}$(OH)$_{22}$ significantly activated primary mouse macrophages and produced pro-inflammatory cytokines, evidencing the possible role of these fullerenols in antitumor immune response.

9. Future and perspectives

It is evident from the biological and medical applications of functionalized empty and endohedral fullerenes that many studies including toxicity and solubility should be addressed. However, many of the biological experiments are far from practical applications at this point. Most of these fullerene derivatives have yet to prove their efficacy and safety *in vivo*, as most have been conducted *in vitro* with a non-consistent variety of cell types. The compatibility of fullerene derivatives with various biological systems ought to be established before any clinical studies are attempted.

One of the biggest obstacles for the progress of fullerene-based treatments is the elevated cytotoxicity due to their hydrophobic nature, which increases their likelihood of absorbing biological species or accumulating in lipid membranes. However, multiple research studies have presented evidence against this fear, or demonstrated that it can be circumvented via modification with biocompatible moieties, as described in this review. Varied cytotoxicity levels and properties are expected for the wide breadth of fullerene derivatives presented in this summary, as their cytotoxic effects depend on different physical properties (size and shape), fabrication methods, and surface functionalization. Systematic toxicological and pharmacokinetic investigations of individual fullerene derivatives are expected to continue and become a more important topic in the field of biomedicine. Beyond studying their therapeutic effects, bioaccumulation studies are just as vital for their eventual translational use.

Conclusions

In this short review we have summarized the biological application of empty as well as of reported endohedral fullerene derivatives during the last 3 years, which include anti-viral

agents, PDT, antioxidants, anticancer, immunological properties, MRI contrast agents and tumor therapy. Remarkable progress has been made on biological applications of fullerenes since the first report in 1993. To solve the almost complete insolubility in aqueous media, several synthetic approaches have been developed as discussed in this review.

Given the fast growth of these compounds for different biological applications combined with low toxicity, it is anticipated that many more biomedical applications will be forthcoming for these interesting compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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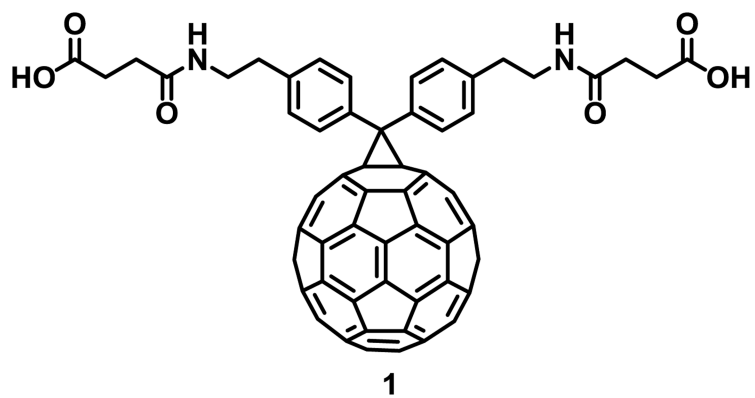
Fig. 1.
First water-soluble and biologically active fullerene derivative reported.
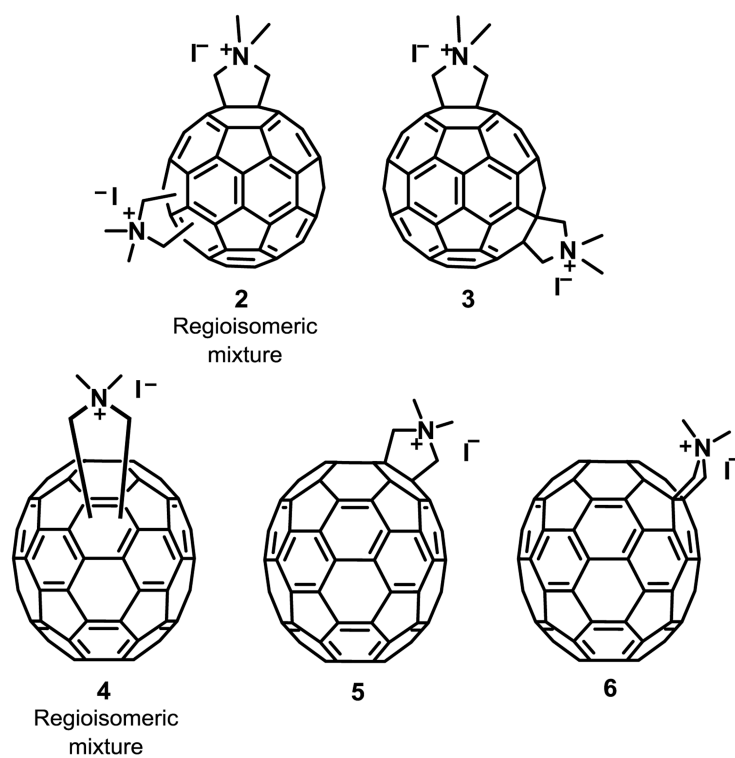
Fig. 2. Maturation inhibitor fullerene derivatives
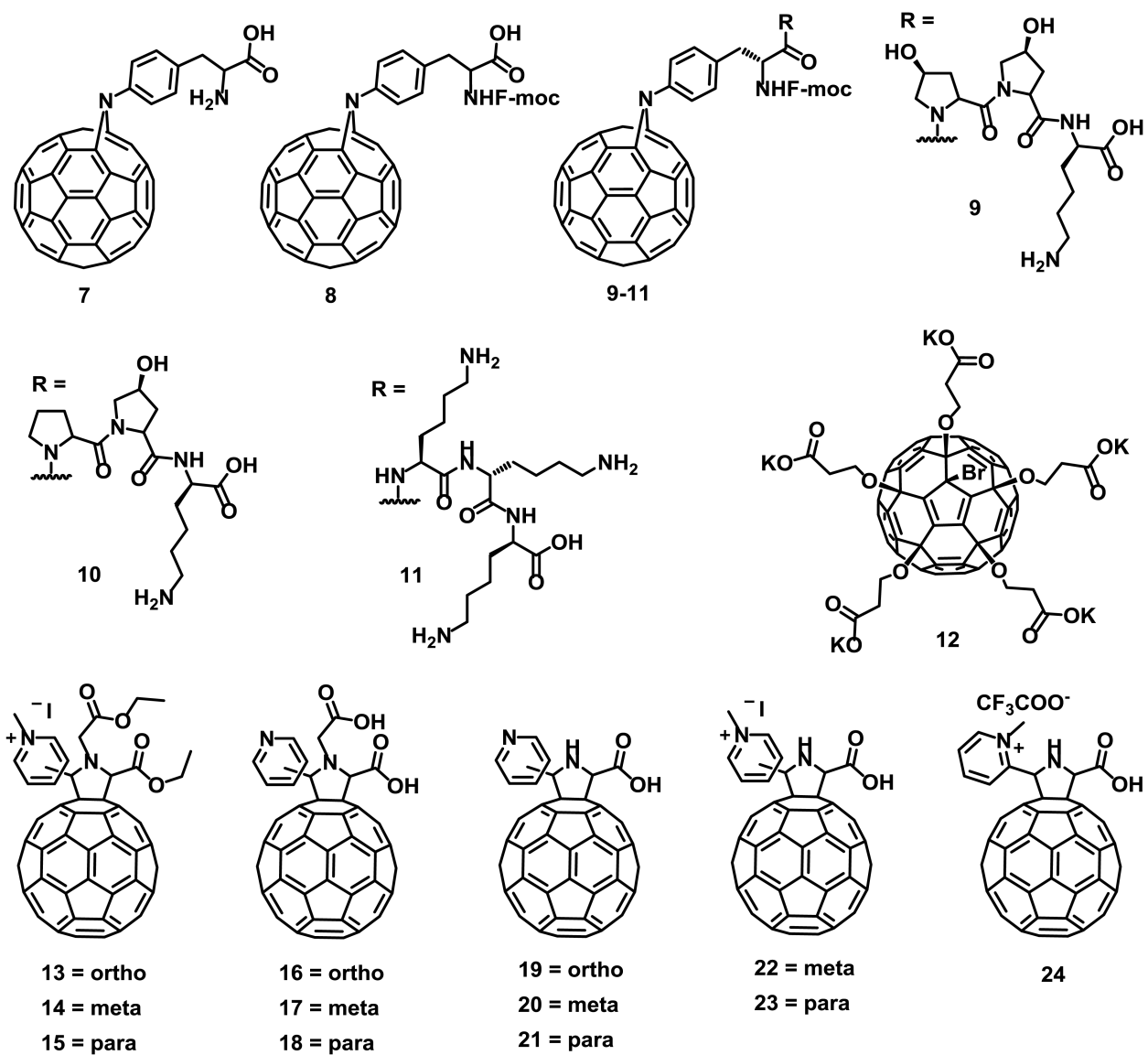
Fig. 3.
Structure of the anti HIV-1 [60]fullerene derivatives **7-24**.
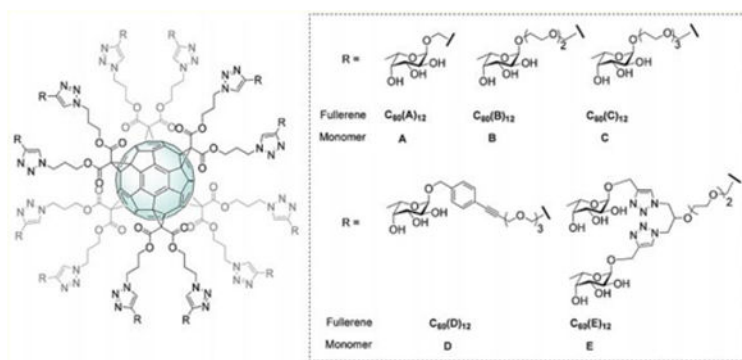
Fig. 4.
Fucofullerenes and their corresponding monomeric controls. Reproduced from Ref.[56] with permission from The Royal Society of Chemistry.
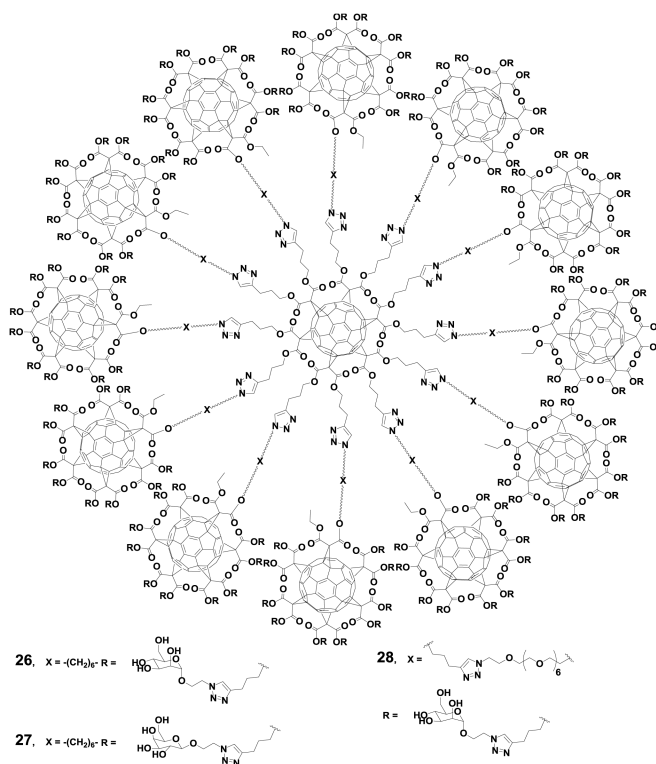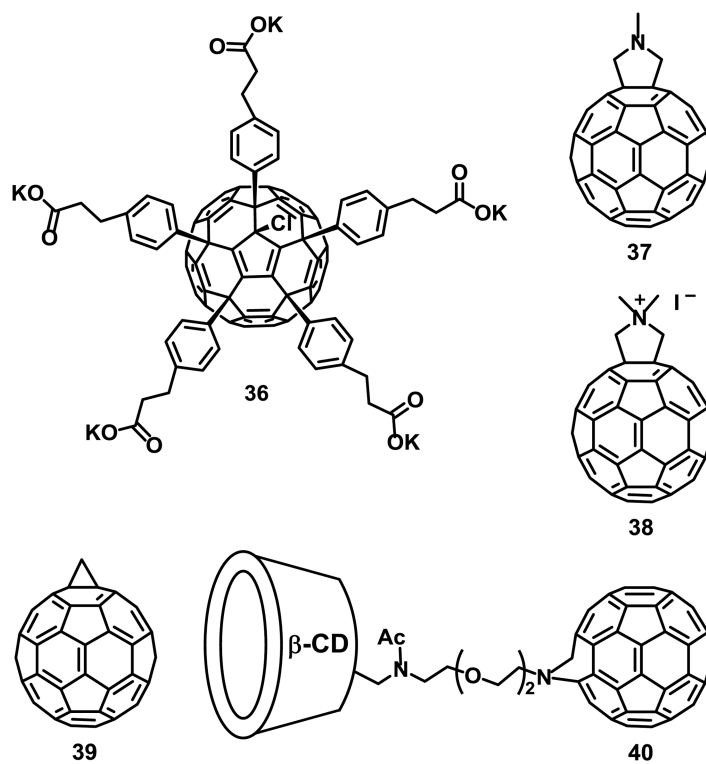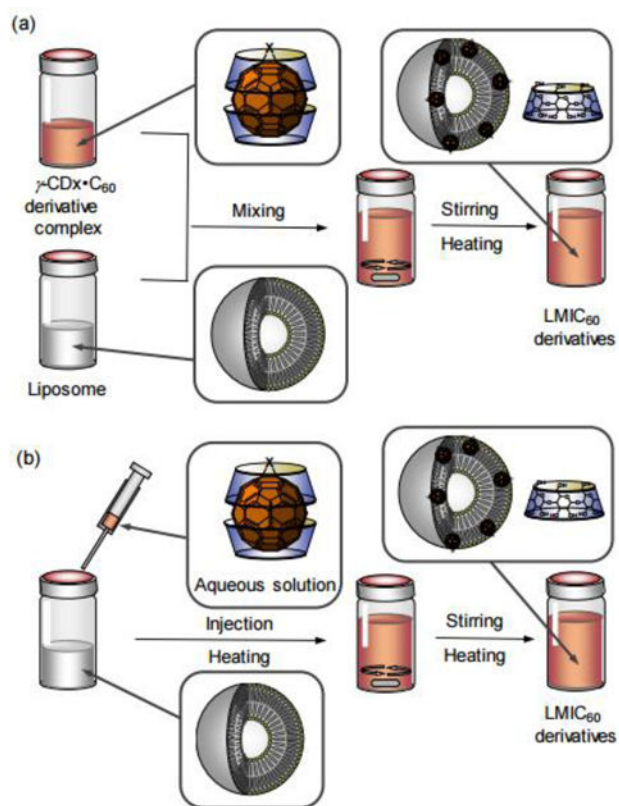
Fig. 5.
Giant globular multivalent glycofullerenes. Reprinted by permission from Macmillan
Publishers Ltd: Nature Chemistry, Muñoz *et al.*[12] copyright (2016).
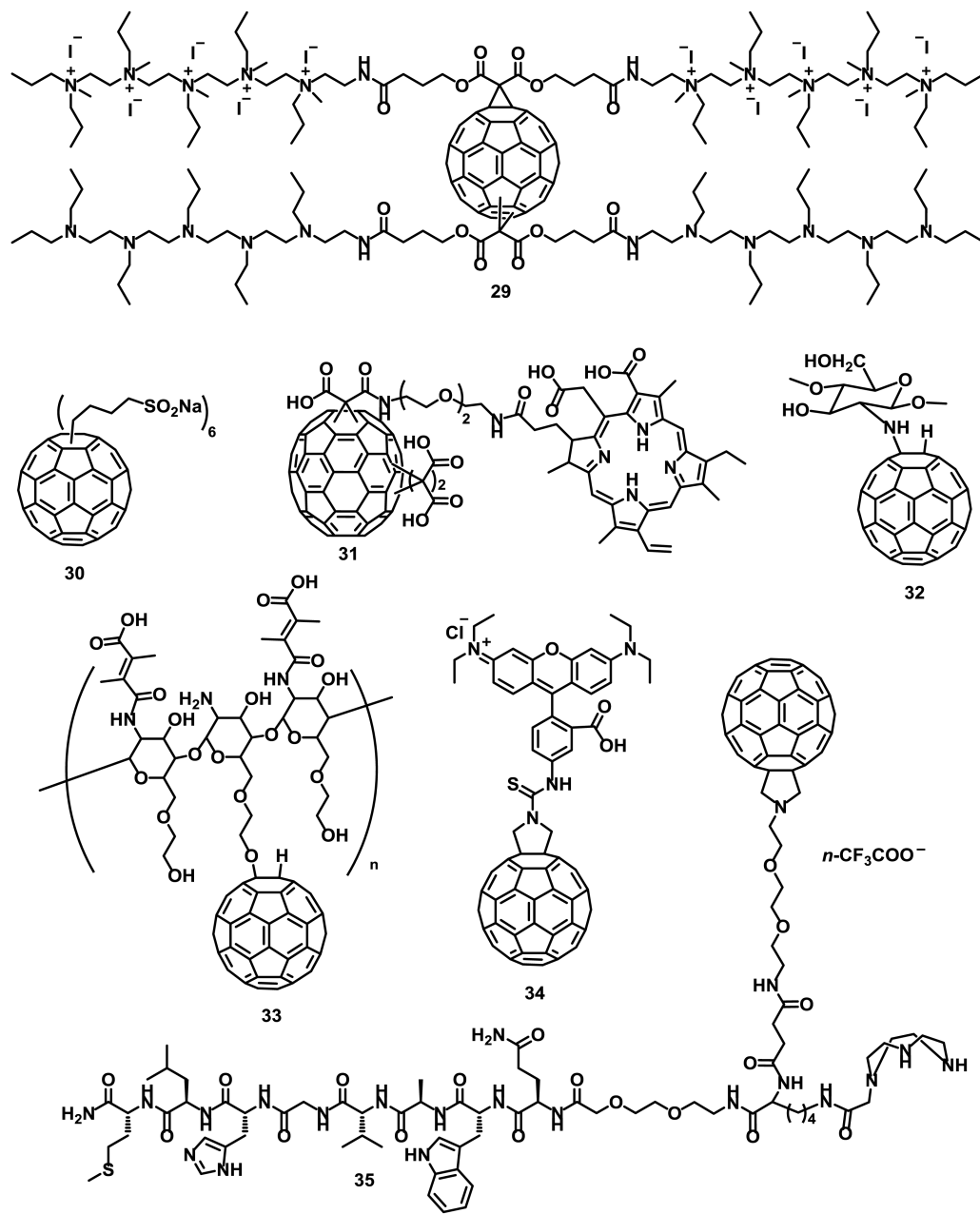
Fig. 6.
Fullerene derivatives for PDT applications.

Fig. 7.
Fullerene derivatives for photodynamic therapy.

Fig. 8.
Exchange method used for the preparation of the $LMIC_{60}$ derivatives. a) Rapid mixing of two solutions and b) Injection. Reproduced from Ikeda *et al.*[21] with permission from The Royal Society of Chemistry.
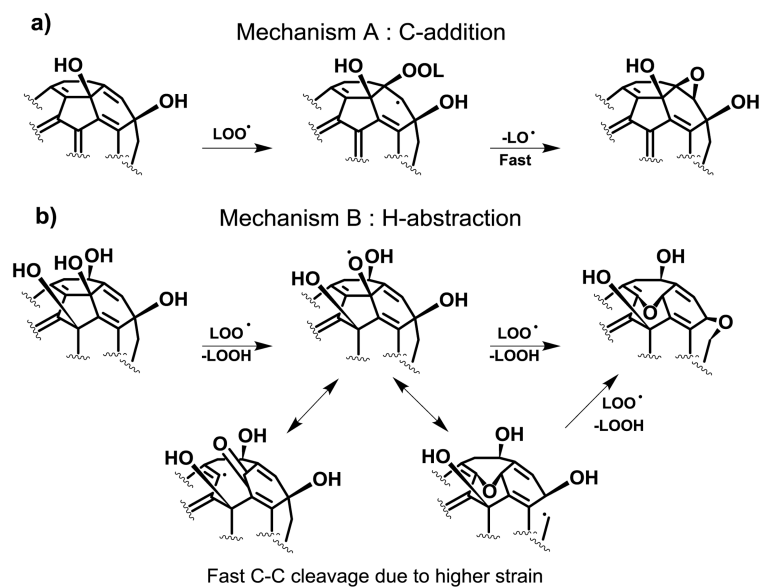
Fig. 9.
Possible mechanisms for lipid peroxyl radical (LOO$^\bullet$) scavenging by fullerenols $C_{60}(OH)_n$: a) addition to C=C bonds and b) H-abstraction from -OH groups. Adapted from Ueno *et al.*[100] (2014).
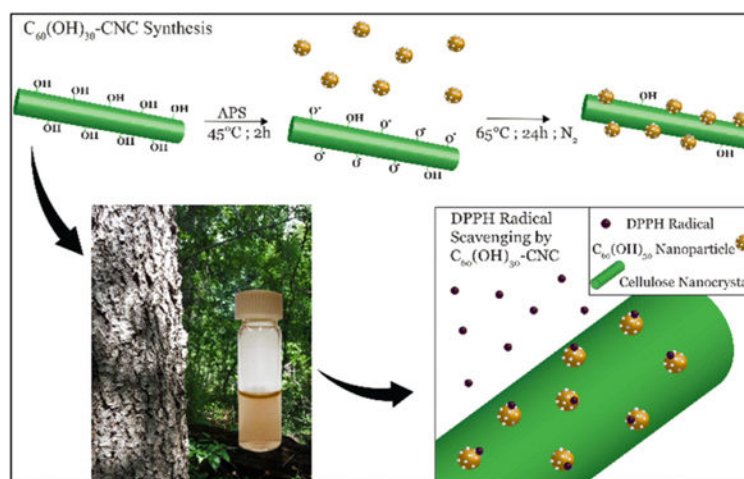
Fig. 10.
Schematic illustrating the conjugation of fullerenols on CNC. Reprinted from Awan *et al.*[104] (2016), with permission of Springer.
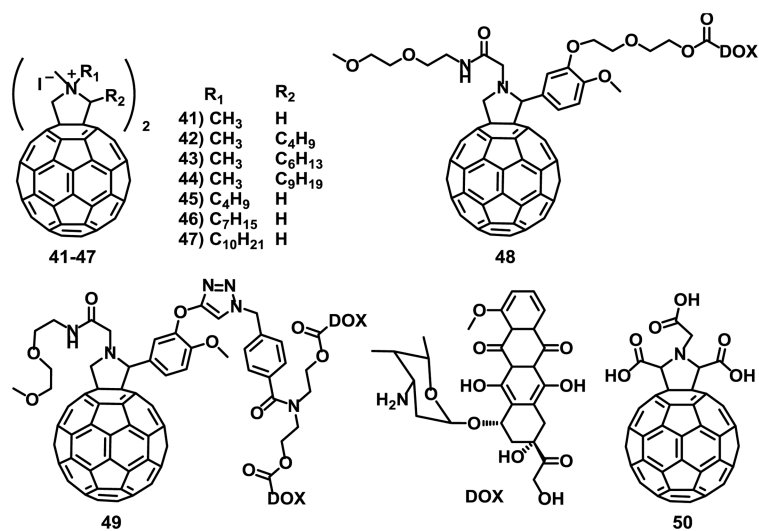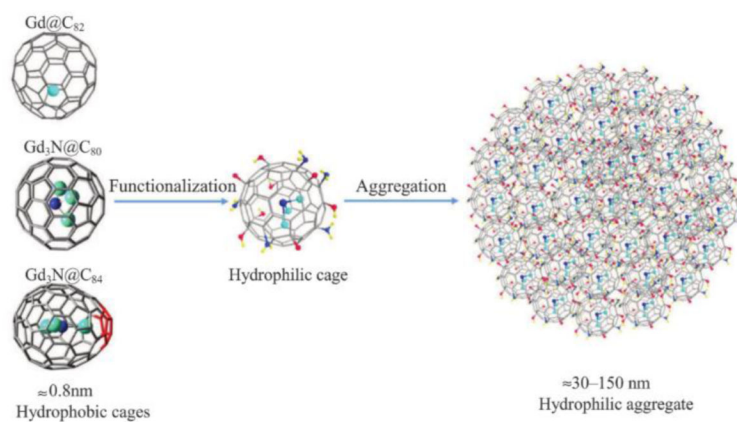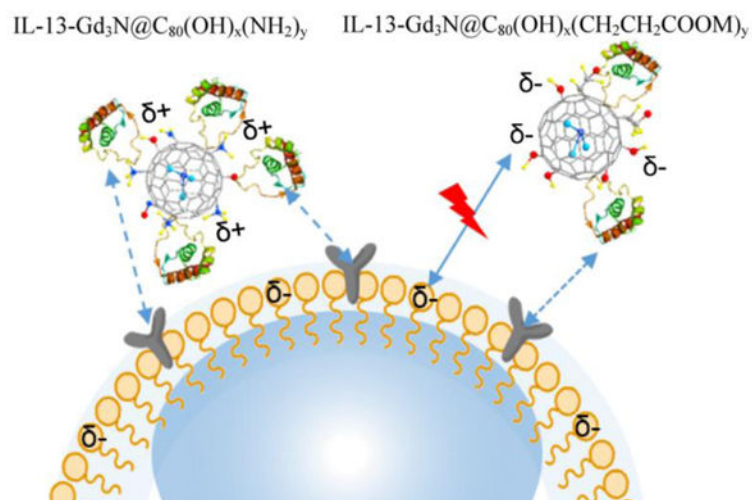
Fig. 11.
Fullerene derivatives with anticancer properties.
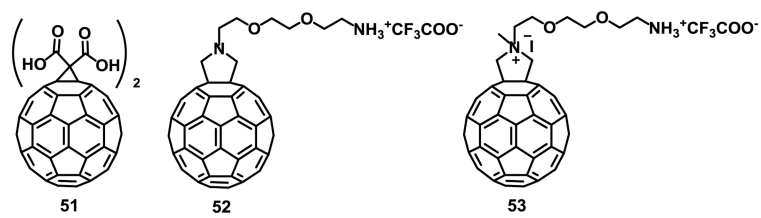
Fig. 12.
Fullerene derivatives with immunological properties.

Fig. 13.
Functionalization of Gd-EMFs. Reprinted with permission from Li *et al.*[130] Copyright (2016) Wiley-VCH and Sons.

Fig. 14.

IL-13-Gd$_3$N@C$_{80}$(OH)$_x$(NH$_2$)$_y$ nanoparticles with positive charges illustrating facile binding to a negatively charged phospholipid bilayer cellular surfaces. Reprinted with permission from Li *et al.*[139] Copyright (2015) American Chemical Society.
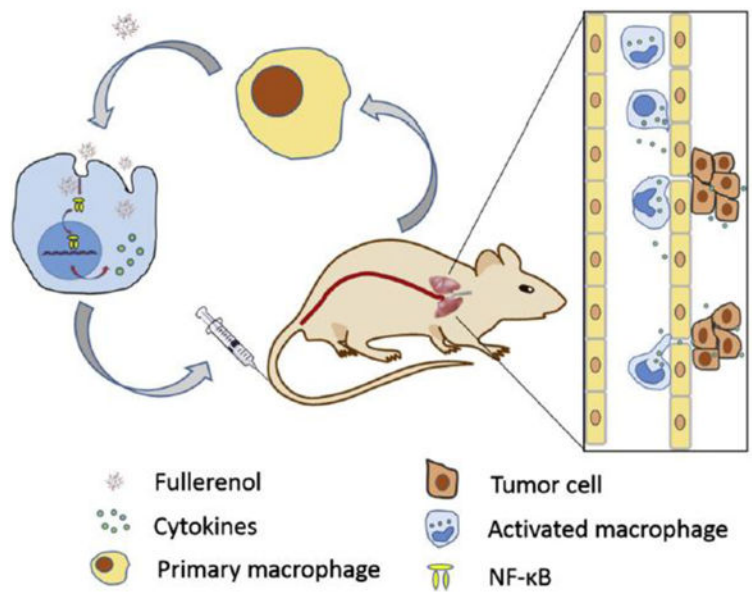
Fig. 15.
The possible mechanism by which back transfusion of macrophages activated by Gd@C$_{82}$(OH)$_{22}$ nanoparticles inhibit tumor metastasis. Reprinted from Tang *et al.*[142] Copyright (2016), with permission from Elsevier.