

Invited Review

Fullerene derivatives: an attractive tool for biological applications

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Abstract

The fullerene family, and especially C₆₀, has very appealing photo-, electro-chemical and physical properties, which can be exploited in many and different biological fields. Fullerene is able to fit inside the hydrophobic cavity of HIV proteases, inhibiting the access of substrates to the catalytic site of the enzyme. It can be used as radical scavenger; in fact some water-soluble derivatives are able to reduce ROS concentrations. At the same time, if exposed to light, fullerene can produce singlet oxygen in high quantum yields. This action, together with the direct electron transfer from excited state of fullerene and DNA bases, can be used to cleave DNA. In this review we report the most recent aspects of fullerene biological applications.

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Chemical and physical features of C₆₀, the most representative among fullerenes, together with his spherical shape, unequalled in nature, have aroused the hope of a successful use in many different fields either in biological and material chemistry. The fullerene interesting properties have stimulated the synthesis of a large number of compounds with different targets, achieving promising results [1–4].

Negative aspects of these molecules for use in medicinal chemistry, like the well-known lack of solubility in polar solvents and the consequent formation of aggregates in aqueous solutions, can be solved by means of chemical or supramolecular approaches. Computational studies on C₆₀ solubility in 75 organic solvents were performed to develop quantitative structure–solubility relationships [5] and different methods to overcome these problems have been extensively explored.

Fullerenes can be entrapped in cyclodextrins [6–8] and calixarenes [9–14]; they can be cosolved with PVP in chloroform [15] or incorporated in suspensions, mixing water to a benzene solution added by acetone

and tetrahydrofuran [16] or into artificial lipidic membranes [17].

The most versatile methodology is based on the chemical modification of the sphere that leads to a wide variety of C₆₀ derivatives, possessing different physical and chemical properties [18].

The best result so far has been obtained by Hirsch and coworkers, who synthesized a dendrimeric fullerene derivative bearing 18 carboxylic groups (Fig. 1), reaching a high level of solubility in water (34 mg mL^{−1} at pH 7.4) [19].

Samai and Geckeler [6] and Filippone et al. [20] developed an elegant approach, covalently linking C₆₀ to cyclodextrins obtaining water-soluble fullerene derivatives, in which the presence of cyclodextrin and its ability to mask the fullerene sphere play an important role in enhancing solubility in polar media and in decreasing aggregation phenomena.

Various functionalizations have been utilized both to increase the hydrophilicity (e.g. –OH, –COOH, –NH₂) and to prepare new compounds presenting biological and pharmacological activity. New ways of action can be induced by the presence of addends on the carbon cage but, in general, the new derivatives are related to the fullerene physical and chemical properties. The lipophilicity of the sphere can be helpful for interactions with the active site of various enzymes, HIV protease

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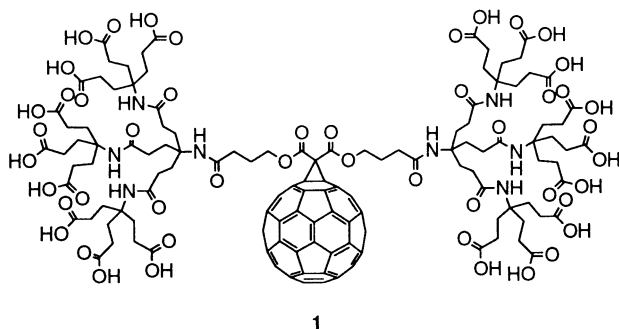


Fig. 1.

(HIVP) among them [21,22], or can make C_{60} able to intercalate into biological membranes, destabilizing them. These actions could have a role in the antibacterial activity found for several derivatives [23]. For its electrochemical features, the fullerene core can react with free radical species behaving as radical sponge in diseases caused by a hyper-production of reactive oxygen species (ROS) [24]. On the other side, C_{60} is able to generate singlet oxygen after irradiation and this can be used to cleave nucleic acids and to oxidize lipids.

It is possible to entrap metal atoms into the fullerene cage obtaining endohedrals metallofullerenes, which can be useful as radiotracers in magnetic resonance and X-ray imaging (MRI and XRI). Fullerenes could be also used as drug carriers for a selective tissue targeting.

In this review we briefly summarize the recent developments achieved in this very active field of C_{60} chemistry.

1. Fullerenes as antioxidants and neuroprotective agents

Many neurodegenerative disorders such as Parkinson's, Alzheimer's and Lou Gehrig's diseases are due to the hyper-production of oxygen and nitric oxide radical species probably due to the over-excitation of glutamic acid receptors.

Oxidative stress by oxygen radicals is known to induce cellular instability by a cascade of events, leading to a programmed cell death. In these situations the use of radical sponges has been demonstrated to decrease, yet not to eliminate, neuronal death. The neuroprotective activity of fullerenes is based on their capability to react with oxygen radical species such as superoxide ($O_2^{\bullet-}$) and hydroxyl ($\bullet OH$) radicals, which attack lipids, proteins, DNA, and other macromolecules. In particular, poly-hydroxylated fullerenes named fullerolols or fullerols [$C_{60}(OH)_n$] have been shown to be excellent antioxidants, reducing apoptosis in cortical neurons cultures: with their high solubility and their ability to cross the blood brain barriers, fullerols have been also demonstrated to absorb many oxygen radicals per fullerene molecule and to reduce the toxicity of free

radical damage on neuronal tissue [25]. They have also shown to block glutamate receptors and to lower the intracellular calcium concentration. AMPA receptors are affected more than NMDA and KA receptors and the inhibition is dose-dependent with a reduction of neurotoxicity of about 80% at 50 μM [26].

Carboxyfullerenes **2** and **3** (Fig. 2) [27] have demonstrated their efficacy *in vivo* toward neurodegeneration involved in the amyotrophic lateral sclerosis (ALS) [24] and they prevent oxidative stress induced by iron in the dopaminergic nigrostriatal pathway [28].

Corona-Morales et al. reported the positive action of compound **2** and of ascorbic acid when applied to adrenal chromaffin cell cultures exposed to levodopa, increasing the cell survival and preventing cell death, including apoptosis [29].

Wang et al. [30] compared the antioxidant power of C_{60} , derivatives **2**–**4** (Fig. 2), and tocopherol (vitamin E) against 1O_2 and $\bullet OH$, respectively generated from the enzymatic xanthine/xanthine oxidase and Fenton reactions. The C_{60} resulted to be the most effective among the considered liposoluble compounds (C_{60} , **4**, tocopherol). A different behavior between the water-soluble isomers **2** and **3** was observed. The C_3 (compound **2**) was more active than D_3 and this can be explained noticing that, in C_3 , all the carboxylic groups are on the same hemisphere, allowing the insertion of C_{60} into the double lipidic layer.

Compound **4** has been also used *in vivo* and by intravenous administration it does not induce protection of cortical infarction. On the contrary, after intracerebro-ventricular infusion, there is not only attenuation of cortical infarction, but also prevention of lipid peroxidation [31].

$C_{60}(ONO_2)_{7\pm 2}$ have been used to attenuate ischemia reperfusion induced lung injury by antioxidant action [32] and other compounds, such as a water-soluble hexasulfonated derivative, have demonstrated to be effective against oxidative stress *in vivo*. The chronic pretreatment of gerbils reduce infarct volume after induced focal cerebral ischemia [33]. In other experiments the same compound was shown to decrease lactate dehydrogenase blood concentration and to increase NO content in plasma after intravenous administration [34]. Hexa(sulfobutyl)fullerene derivatives have

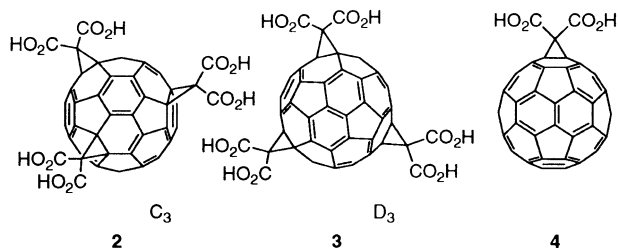
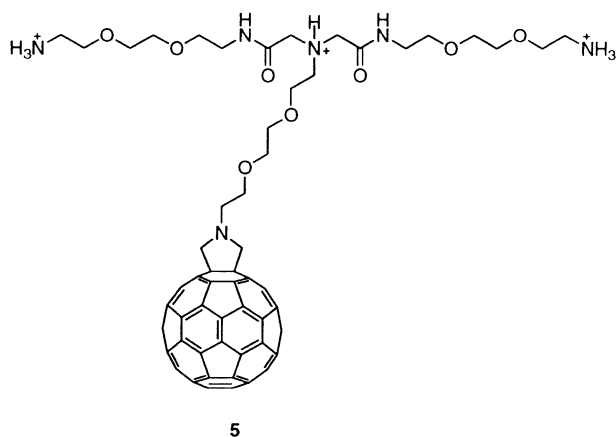


Fig. 2.

been studied as antiproliferative agents in the arteriosclerosis for their ability of inhibition plasma lipid peroxidation acting as radical scavenger [35,36].

Very recently, our group reported the synthesis of a water-soluble mono-substituted C_{60} derivative (**5**), with the aim of investigating its radical scavenging properties. This compound was tested to prevent cells from glutamate-induced neuronal death by *in vitro* assay. However, this compound proved to be highly toxic inducing itself cell death. This behavior can probably be attributed to the amphiphilic properties of this compound, which, after interactions with cell membranes, induces cell disruption. This experimental observation suggests that oligo- or polyfunctionalized C_{60} derivatives should be better neuroprotective agents than monofunctionalized fullerenes, despite their minor loading of ROS with respect to fullerene monoadducts [37].



2. Antiapoptotic activity

Apoptosis is a scheduled cell death that is mainly due to the transforming growth factor (TGF- β), a dimeric protein of 25 kDa. In this process, ROS species are released and one way to stop the damage, or at least to decrease it, is an antioxidant treatment. Huang demonstrated that fullerene derivatives **2** and **3** can prevent apoptosis in hepatic tumor cells Hep3B by neutralization of the TGF- β induced reactive oxygen species [38]. Further experiment supports the antiapoptotic effects of hexa(sulfobutyl)fullerene derivative in kidney cells exposed to an oxidative stress induced by ischemic event and following reperfusion (I/R) [39]. The prolonged lack of oxygen followed by an organ reperfusion gives rise to a sequence of cell modifications that culminate in apoptosis. The decrease of apoptotic cell death level is strictly related to the neutralization of ROS both *in vitro* and *in vivo*. Compound **2** is able to reduce anoikia, apoptotic cell death induced by growing substrate deprivation, on epithelial cells [40] and its effect was

studied also on cerebellar granule cells and on peripheral blood mononuclear cells (PBMC) [41,42]. In this case 2-deoxy-D-ribose or TNF- α plus cycloheximide were used to induce apoptosis and carboxyfullerene resulted to prevent their actions on PBMC.

3. DNA photocleavage

From a considerable number of studies, nucleotidic chain cleavage can be performed in the presence of fullerene derivatives. This phenomenon takes place only in the presence of light and was studied on animal microbial cells lines (Salmonella) and on plasmides (pBr322). In both cases the fragmentations of the DNA and RNA filaments were observed [43].

There are two different pathways of DNA photocleavage acting mainly at guanine sites: it has been demonstrated that generation of singlet oxygen as well as energy transfer from the triplet state of fullerene to bases can be responsible of the oxidation of guanines [44,45]. Both mechanisms are related to photoexcitation of fullerene. 1O_2 acts by [4+2] or [2+2] cycloaddition on G and these modifications increase the instability of the phosphodiesteric bond that becomes easily susceptible of alkaline hydrolysis. The triplet state of fullerene forms by intersystem crossing from the singlet state, produced by photoirradiation. The triplet state can lead to the formation of intra- and intermolecular charge transfer complexes among C_{60} and aromatic rings or tertiary amines [46,47]. The fullerene molecule could be free or complexed with a solubilizing agent, in fact Ungurenasu and Airinei demonstrated that C_{60} -PVP complexes have the ability to form very easily charge transfer complexes keeping their solubility in water [48].

Conjugations of fullerene with molecular fragments that interact with DNA as intercalators or minor groove binders have been performed, increasing the affinities with the nucleic acids. Compounds with a fullerene core covalently linked to a DNA portion should improve the action and the selectivity versus target DNA. A very promising approach has been utilized by Boutorine et al., who synthesized a compound bearing a short oligonucleotide chain able to interact either with a single filament or with a double chain, forming a duplex or a triplex respectively, following the same process of antisense nucleotides [49]. Analogous approach has been developed by An et al. [50]. Recently, in our laboratory, new hybrid derivatives, bearing both oligonucleotide chain and a trimethoxyindole (TMI) unit used as a minor groove binder, have been synthesized, with the aim to increase potency, selectivity and stabilization of the triple helix (compounds **6** and **7**, Fig. 3) [51,52]. This new approach needs some improvement, in particular the optimization of the spacer link between TMI and C_{60} , because of steric and electrostatic interactions, that

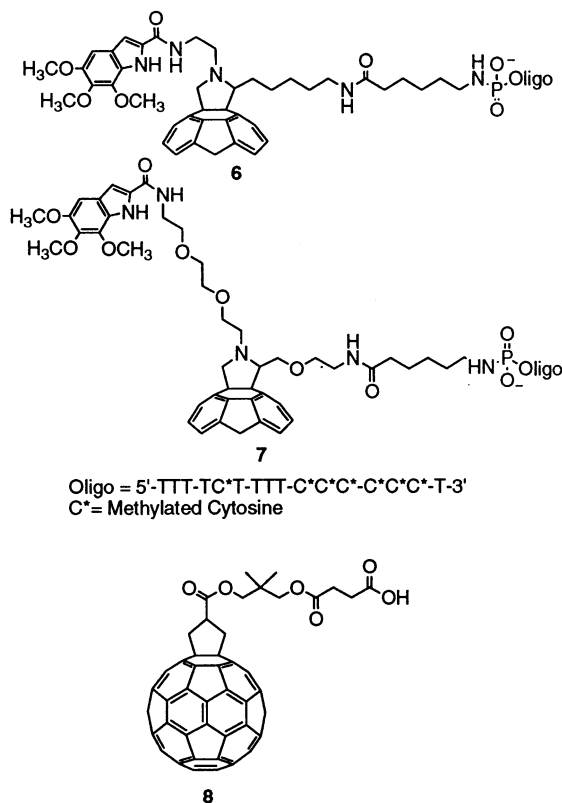


Fig. 3.

do not permit an optimal interaction with the double stranded DNA.

The main direct consequence of nucleic acids cleavage is cytotoxicity, observed in many tissues. *In vitro* studies on hepatic tumor cells (HeLa S3) showed that compound **8** is active only after irradiation.

Mutagenicity phenomena in *Salmonella* tester strains have been revealed together with inactivation of Togaviridae and Rhabdoviridae viruses [53]. It was also possible to immobilize DNA on a monolayer whose ends consist in cationic groups able to link the double helix phosphates, already intercalated by C₆₀. Irradiation of the monolayer led to site-specific cleavage of the nucleic acid: it should be possible to use fullerene and its derivatives as photoprobes in the study of genic transcription [54].

4. Enzyme inhibition

Fullerene derivative **8** (Fig. 3) has shown inhibitory activity against various enzymes as cysteinic proteases (papaine, cathepsine) and serinic proteases (trypsin, plasmin, thrombin) [43].

The unique characters of hydrophobicity and electrophilicity together with the high reduction potential are probably the key elements for this activity but the mechanism is still unknown. Recently inhibition to-

wards glutathione-S-transferase has been reported [55] and, in the case of fullerols, towards P450-cytochrome-dependent monooxygenases, plasmatic reticulum enzymes of hepatic cells and mitochondrial ATPase in the process of oxidative phosphorylation [56].

A relevant development in enzyme inhibition by fullerene derivatives relates to nitric oxide synthase (NOS). Nitric oxide is a very reactive radical molecule and it is an important physiological, almost ubiquitous messenger. However at high concentrations it becomes toxic. After the demonstration that fullerols are able to decrease bronchospasm induced by the system xanthine/xanthine oxidase [57,58], inhibition of all the three forms of NOS, neuronal, epithelial and inducible, has been found by trimalonic derivatives of C₆₀, mainly C₃ and D₃ (Fig. 2). The inhibition is multisite and positively cooperative and it seems that C₃ inhibits the inter-subunit transfer of electrons, presumably by a reversible distortion of the dimer interface [58].

5. Anti-HIV activity

In spite of the enormous advances achieved in the therapy of AIDS, the quick mutation of human HIV that leads to resistance toward the modern therapies and the high toxicity of currently used drugs strongly support the discovery of new agents.

HIV protease is a fundamental enzyme for the virus survival. It is an aspartic protease enzyme similar to mammalian proteases like renin, but is specific for HIV proteins and does not cross-react with human proteases. The HIVP cleaves a polyprotein shortly after viral budding. The effect of this cleavage is to activate reverse transcriptase (RT), RNase H, integrase, and protease itself. The latter completes the life cycle of HIV-1 and without this step it is not possible to infect new CD4 cells. The protease was predicted to be one of the main possible targets of the antiviral therapy and at the moment molecules that are mostly HIVP inhibitors are used in clinical medicine. Despite this success however, there is a need for new protease inhibitors, due to the common problems of resistance, which is caused partly by the high tendency to undergo rapid and frequent genetic mutation of the virus, partly to the chemical similarity among clinically available inhibitors.

The HIVP active site is a semi-opened hydrophobic ellipsoid; two aminoacidic residues, 25 and 125 aspartate, stand out on the surface of the cavity and catalyze the hydration of the cleavable peptidic bond of the substrate. The diameter of this cavity is around 10 Å, close to the diameter of the C₆₀ sphere. Some years ago Friedman, Wudl and coworkers performed molecular modeling studies [22] and they found out that HIVP could be complexed and inhibited by the introduction of a C₆₀ molecule into the catalytic cavity. In fact, if the

carbon sphere is perfectly centered, the distance between its surface and that of the enzyme allows the formation of van der Waals interactions.

The same research group performed the first synthesis of a fullerene inhibitor of HIVP (compound **9**). The compound showed to fit smartly in the active site of the viral protease with strong van der Waals interactions on the enzyme hollow surface [21].

In vitro studies were performed on compound **9**. Dissociation rate values with HIVP were around 10^{-6} – 10^{-9} M and the inhibition constant 5.3 μ M. The level of inhibition of acutely infected peripheral blood mononuclear cells (PBMC) resulted in an EC_{50} of 7 μ M and non-cytotoxic effect was detected up to 100 μ M [59].

The introduction in a proper position of substituents able to bring about electrostatic interactions with the two aspartates, 25 and 125, could raise the binding constant up to 1000 times. A guide-line for the synthesis of more efficient molecules is based on a better exploitation of the hydrophobic cavity of HIVP with an increased surface desolvation and with the creation of stronger bonds [60]. In fact, side chains of compound **9** do not play a role in the interaction but they lean forward on the outer side and have only a solubilizing function. A deeper analysis of the enzyme surface revealed the presence of two symmetrical channels that are exposed to solvation and cannot be occupied by the side chains of **9**. The addition of non-polar groups, able to insert in those regions, could increase desolvation. Derivatives **10**–**12** (Fig. 4) studied by Friedman et al. [61] can strongly enhance the desolvation and partially occupy the water-exposed channels. In particular for compound **11** the calculated K_i was 103 nM, 50 times higher than K_i of compound **9**.

Also the fullerene dendrimer **1** was evaluated for the anti-HIV activity. The compound was tested in primary human lymphocytes acutely infected with HIV-1_{LAI}, with an EC_{50} of 0.22 μ M and it demonstrated to be

effective also against mutant viruses. Besides, no apparent cytotoxicity in human PBM, Vero or CEM cells was evidenced up to 100 μ M. Molecular modeling studies showed that also in this case the fullerene core could block the access to the catalytic site. From calculations for the intermolecular energy of the minimize model, the interaction between the fullerene moiety and the enzyme results to be energetically favored [62].

Two compounds (**13** and **14**, Fig. 5) bearing two ammonium groups were synthesized in our group [63]. The distance of the two functional groups is around 5.1 Å, a value very close to the distance calculated for compound **15** hypothesized by Friedman et al. [22], ideal for giving electrostatic interactions with Asp 25 and Asp 125, avoiding the direct regioselective bis-addition to the carbon. For this reason they may exhibit interesting anti-HIV activity. The complexation energies of HIV protease were evaluated and the results seemed very promising.

Toniolo et al. synthesized a peptidic fullerene bearing the C-terminal sequence of peptide T, capable of activating chemotaxis of human monocytes through CD4/T₄ antigen and to inhibit the HIVP activity. Derivative **16** was shown to be a potent agonist of chemotaxis, with activity comparable to the fragment of peptide T alone, active in the range 10^{-10} – 10^{-8} M. Besides it weakly interacts with the active site of HIVP at a concentration of 100 μ M [64].

A very recent work describes molecular dynamics study of the connection between flap closing and binding of fullerene inhibitors of HIV-1 protease [65]. In this paper the authors analyze the hydrophobic desolvation of HIVP cavity in the presence of fullerene derivatives, together with the binding affinity and the

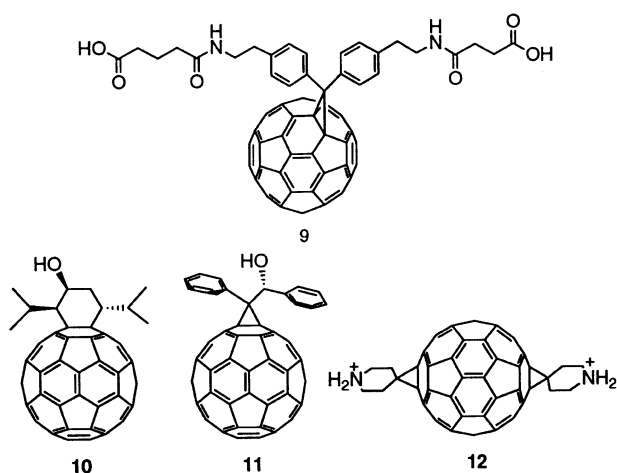


Fig. 4.

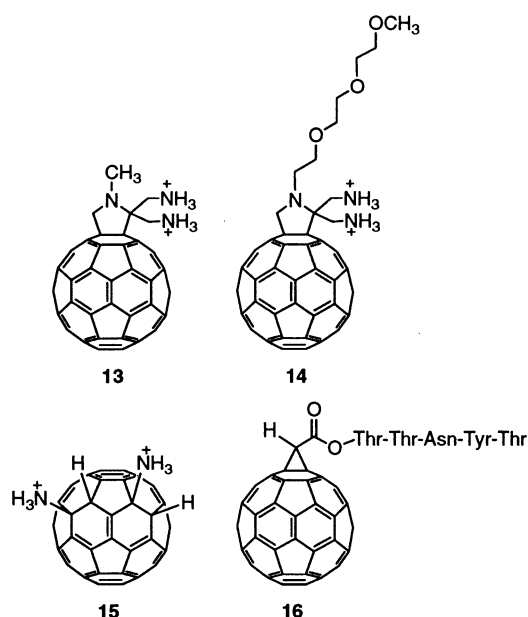


Fig. 5.

flap motion of the enzyme, predicting the tight closing of the flexible flaps when an effective binders is docked into the active site. They found an important exclusion of water from the region between the fullerene and the cavity, particularly in the vicinity of the catalytic aspartate residues. This leads to an increase of hydrophobic interactions between inhibitor and enzyme.

6. Antimicrobial activity

The discovery of the possible intercalation of fullerenes into biological membranes has encouraged many research groups to study the potential antimicrobial effects of C_{60} . Positive results were achieved on bacteria like *Candida albicans*, *Bacillus subtilis*, *Escherichia coli* and *Mycobacterium avium* [66,67]. Particularly interesting was the higher efficacy of salts **18** (Fig. 6) in comparison with respective neutral derivatives **17** even against resistant strains of human *Mycobacterium tuberculosis* [23].

The biological effects of tricarboxyfullerene derivative have been studied on twenty different bacteria strains, as *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus faecalis* (Gram positive); *Klebsiella pneumoniae*, *E. coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* (Gram negative) by Tsao et al., who have demonstrated that all the G-positive are inhibited with a MIC $\leq 50 \mu\text{g mL}^{-1}$, with a minimum of $5 \mu\text{g mL}^{-1}$ for *Streptococcus pyogenes* A-20 [68]. The Gram negative did not have any reaction neither at $500 \mu\text{g mL}^{-1}$. By virtue of the different interactions with Gram positive and Gram negative bacteria, the bactericide action was found to be based on the insertion into the microbial cell wall. Besides, the intercalation of carboxyfullerene into the cell-wall was demonstrated by transmission electron microscopy and anti-carboxyfullerene binding assay. The authors analyzed more in detail the action on *Streptococcus pyogenes* A-20 infection [69]. The death induced by this bacterium can be inhibited by carboxyfullerene in dose-dependent manner, with protection of the 33% of mice from death. The proposed mechanism implies an enhanced bactericidal activity of the neutrophils.

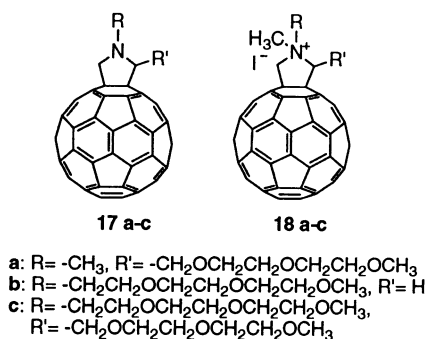


Fig. 6.

The same research group found that carboxyfullerene is able to inhibit *E. coli*-induced meningitis. The production of TNF- α and IL-1 β together with the inflammatory neutrophilic infiltration and the blood–brain barrier (BBB) permeability resulted to be decreased in mice treated with fullerene derivative with respect to controls [70]. This protective action mediated by fullerene seems to not involve the direct inhibition of the microbial growth. In fact it seems that fullerene can inhibit the late phase of BBB permeability, the neutrophil-associated BBB opening [71].

A different group of fullerene derivatives with good potential antibacterial activity is represented by fulleropeptides [72]. This class of compounds has already a role in biological application of fullerene (see anti-HIV activity) but new approaches and new possible applications are now under consideration. The fullerene has been used for the first time in the solid phase peptide synthesis (SPPS) to prepare peptide derivatives [73]. This approach permits to utilize aminoacidic C_{60} derivative **19** as building block in the preparation of peptides by direct utilization of SPPS. Compound **20** (Fig. 7) combines the lipophilic properties of fullerene with the water-solubility and electrostatic interactions conferred by the peptide portion and shows interesting antimicrobial properties. In fact it was tested on *S. aureus* and *E. coli*, with MIC of 8 and $64 \mu\text{M}$, respectively. The corresponding non fullerene–peptide **21** did not show any activity and this can suggest an important role of amphipaticity in antimicrobial action.

In the preparation of other antibacterial peptides, compound **19** has been used at the N-terminus of the peptide or in the middle or at the COOH-terminus [74]. Actually a peptide called P19(8) was utilized, known to be active against Gram negative (i.e. *E. coli*) and yeast (*C. albicans*) but presenting low activity versus Gram positive (*S. aureus*). The introduction of fullerene core induced a remarkable effect on antibacterial activity and

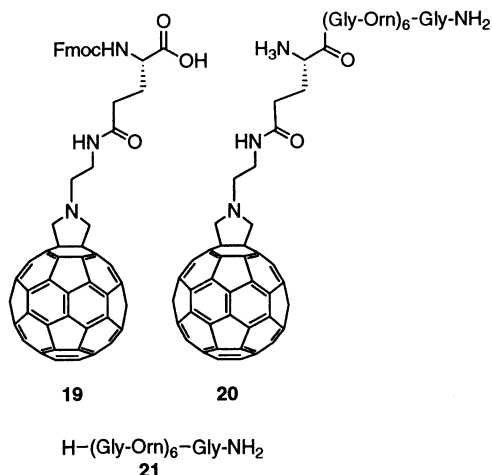


Fig. 7.

also on hemolytic activity. The general behavior consists in an increase of potency versus Gram positive and in a reduction of effectiveness against Gram negative and yeast [74].

Different mechanisms, not involving the cellular membrane disruption by intercalation of the lipophilic fullerene moiety, can be utilized to inactivate pathogen agents. Mashino et al. [75] utilized two isomers (*trans*-2 and *trans*-4) of C_{60} -bis(*N,N*-dimethylpyrrolidinium iodide) (compounds **22** and **23**, Fig. 8) to study the bacteriostatic effects of fullerene derivatives on *E. coli* and they attribute this to the inhibition of energy metabolism, by two opposite dose-dependent mechanisms. At low fullerene concentration the oxygen uptake is decreased, on the contrary at high concentration the O_2 uptake is increased and oxygen is converted to H_2O_2 . Besides, these fullerene concentrations inhibit the respiratory chain activity [75].

7. Fullerenes in the osteoporosis therapy

Osteoporosis is a serious health problem since 25% of women over age 65 present one or more vertebral fractures caused by osteoporosis. These fractures are associated with a mortality rate of 15%. Indeed, falls are the leading cause of accidental death in the elderly, primarily because of hip fractures.

It is well established that bisphosphonate compounds are bone-active and bone-seeking drugs useful in the treatment of osteoporosis and other bone disorders. In addition, the F^- anion is the only known agent that can generate new bone matrix and new mineral from previously inactive areas. As such, F^- is also useful in the treatment of osteoporosis where it improves bone strength and helps prevent fractures. However, bisphosphonate drugs (orally administered) are not efficiently absorbed in the gastrointestinal tract, while the F^- anion (orally administered as NaF) is fairly toxic. In general, there is a need for more effective and less toxic bone-active drugs, particularly bisphosphonates and F^- or their equivalents.

Bone tissue is an especially appealing target for vectored pharmaceuticals because its primary inorganic component, hydroxyapatite (HAP), offers a multitude

of binding sites for structurally suitable compounds. Substances with functional groups such as hydroxyl groups and carboxylic and phosphonic acids are capable of forming ionic and hydrogen bonds to the mineral portion of bone [76–78].

Tissue-vectored bisphosphonate fullerene **24**, $C_{60}(OH)_{16}AMBP$ (Fig. 8), designed to target bone tissue has been synthesized and evaluated in vitro [79]. An amide bisphosphonate addend, in conjunction with multiple hydroxyl groups, confers a strong affinity for the calcium phosphate mineral hydroxyapatite of bone. Constant composition crystal growth studies indicate that $C_{60}(OH)_{16}AMBP$ reduces hydroxyapatite mineralization by 50% at a concentration of 1 mM. Parallel studies with $C_{60}(OH)_{30}$ also indicate an affinity for hydroxyapatite, but a reduced level (28% crystal growth rate reduction at 1 mM) compared with $C_{60}(OH)_{16}AMBP$. This study is the first to demonstrate that a fullerene-based material can be successfully targeted to a selected tissue as a step toward the development of such materials for medical purposes.

8. Anti-fullerene monoclonal antibodies

Practical applications of fullerenes as biological or pharmacological agents requires that dosage and serum levels be capable of measurement, preferably by sensitive and simple immunological procedures. This requires that specific antibodies to fullerenes be produced [80]. Hailed as one of the major advances in biotechnology, tailor-made identical copies of antibodies—monoclonal antibodies—have matured to contribute significantly to modern therapeutics. Bioengineering of antibodies either as a whole or as specific binding fragments (Fab) are currently used in the armamentarium of cancer therapeutics and of other proliferative diseases including arthritis.

The immunization of mice with a conjugate between C_{60} and bovine thyroglobulin led to the production of an anti-fullerene antibody series of IgG isotype, demonstrating that the immune system is enough specialized to recognize and process conjugate fullerenes. The resulting antibodies population included a sub-population cross-reacting with C_{70} , as it was visible from immune precipitation techniques and ELISA assays. These tests were possible through the synthesis of new hydrosoluble derivatives, like conjugates between C_{60} and bovine and rabbit serum albumine and trilylsine and pentalyline derivatives [80].

Furthermore, a conjugate between C_{60} and a Fab molecule was synthesized and several antibodies for non- C_{60} -fullerenes have been developed.

It is important to underline that only very small quantities of fullerenes are needed to stimulate an immune response and, therefore, to produce workable

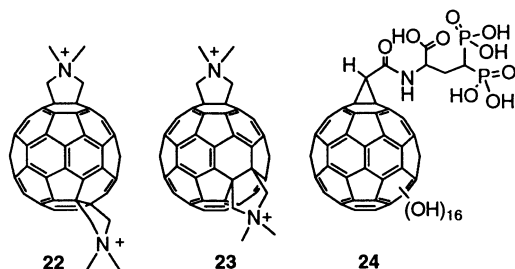


Fig. 8.

amounts of monoclonal antibodies. As fullerene derivatives become useful clinically, anti-fullerene antibodies are ideally suited for serum assays. In this regard, CSixty Inc. has developed the first immunological assay, as a kit, to detect and quantify fullerenes in biological fluids (see <http://www.csixty.com>).

9. Fullerene-based contrast agents and radiotracers

When injected into the blood stream, selected fullerene compounds rapidly distribute into tissues, with no detectable toxicity for at least one week. The distribution and relocation of fullerene compounds in selected organs offer the opportunity for organ-specific targeting. Fullerene compounds therefore offer a unique opportunity for the design of organ-specific drugs.

X-ray contrast agents are used in aqueous solutions that are injected in large quantities (up to 100 mL) by catheterization, followed by immediate imaging. The contrast agents are restricted to the extra-cellular spaces and are rapidly excreted within minutes of injection into the bloodstream. Pulmonary and arterio-angiograms, kidney/bladder images and more can be obtained. Two representative examples of widely used contrast agents are Iohexal and Iopamidol. Although current agents have advanced to the point where they are effective and fairly safe, a portion of the population (2–8%) still experience hypersensitivity (allergic reactions). This results in severe reactions, and, in some cases, death. The mechanisms of toxicity are not fully understood, but lower toxicity has been related to increased ‘sphericity’ of the contrast agent (i.e. non-planar-molecules). In addition, it has been noted that better shielding for the hydrophobic benzene rings by bulky hydrophilic substituents lowers toxicity. Finally, there is a need for contrast agents that can serve as blood-pool agents with an extended retention time, which means slow diffusion in the blood stream. A contrast agent with these properties would be especially desirable for the rapid diagnosis (<0.5 h) of stroke by blood-pool X-ray imaging.

Highly-iodinated C_{60} molecules have been developed representing the foundation of a new X-ray angiography technology in medicine [81]. Demonstration that these iodinated derivatives of C_{60} linger in the blood pool for up to an hour and are relatively non-toxic, will offer the possibility of revolutionizing X-ray angiography by eliminating the catheterization procedure. Long-term blood pool X-ray contrast agents have long been sought as one of the ‘Holy Grails’ of the field, and the molecules herein could be the new paradigm that permits attainment of this goal.

The new X-ray contrast agents based on C_{60} offer the opportunity to reduce toxicity (compared to commer-

cially available X-ray agents) and to produce a unique, new blood-pool X-ray imaging technology.

The entrapment of metal atoms inside the cavity of fullerene sphere leads to the formation of a nanomaterial family called endohedral metallofullerenes [82]. One of the most appealing applications of endohedrals is in the nuclear medicine field. Because of their metabolism resistance and high kinetic stability, they can potentially represent a unique alternative to chelating compounds. The main disadvantage of current drugs containing chelated metal radioisotopes is their pronounced kinetic instability in vivo, which can lead to the release of small quantities of toxic radiometals [83,84].

The toxic heavy radiometal will not be able to exit once it has been placed inside the fullerene cage. The studies of the bioavailability of radioactive endohedral metallofullerene $^{166}\text{Ho}@C_{82}(\text{OH})_x$ confirmed the possibility of using these molecules as radiotracers in in vivo studies. Furthermore its blood residency time, more than one hour, its almost total clearance and the total absence of acute toxicity in vivo make it very interesting [85,86].

Among endohedral metallofullerenes $\text{Gd}@C_{82}(\text{OH})_x$ demonstrated to be a possible contrast agent in magnetic resonance imaging (MRI) [87,88]. This technique is based on the measure of relaxation time of water protons inside the human body after the exposure of a transversal magnetic field. Contrast agents can enhance the resolution of the image produced for example in case of metastatic cancers [89].

A very recent work on in vivo evaluation of $\text{Gd}@C_{60}[\text{C}(\text{COOCH}_2\text{CH}_3)_2]_{10}$ has been published by Bolskar et al. [90]. They evaluated this very soluble C_{60} derivative as MRI contrast agent and found for the first time a non-reticuloendothelial localization of a water-soluble C_{60} derivative. This unusual behavior results to be very interesting for the development of new fullerene-based drugs. Biodistribution study on $^{99\text{m}}\text{Tc}@C_{60}(\text{OH})_x$ has been performed by Qingnuan et al. utilizing single photon emission computed tomography and gamma counting. It was demonstrated that this derivative distributes in all tissues and has a slow clearance over 48 h except for bone [91].

10. Other applications

In the latest years attempts of using fullerenes in different pharmacological fields have greatly increased together with the studies on its physical and chemical properties.

A very important application reported by Nakamura and coworkers implies the utilization of derivatized fullerene **25** as gene transfection agent (Fig. 9). This derivative bears four positive charges, which permit to bind DNA without recognition of specific bases but

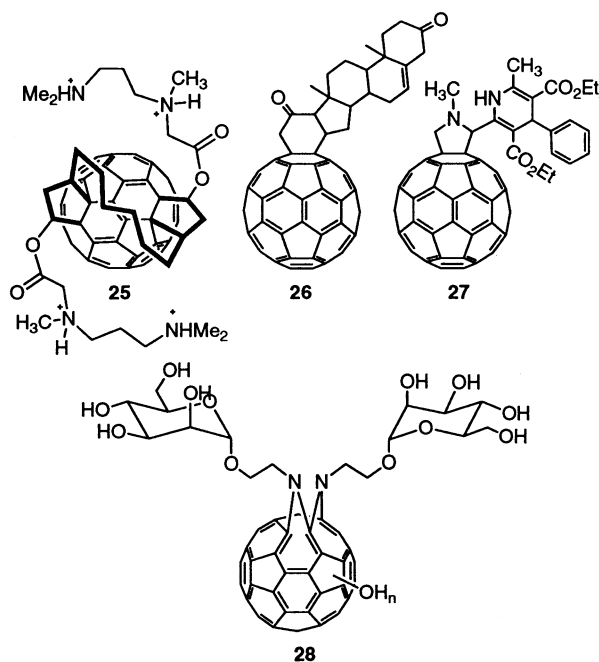


Fig. 9.

thank to electrostatic interactions between the ammonium salts and the negative charged phosphate groups of DNA. The cells take up the complex through phagocytosis and the binding of duplex DNA is reversible [92].

The water-insoluble photosensitizer pristine C_{60} was employed to destroy enveloped viruses by photoirradiation of buffered solutions containing the target virus and fullerene. The activity was demonstrated to be oxygen-dependent but unaffected by the presence of proteins, strongly suggesting the involvement of singlet oxygen [53].

A conjugate between C_{60} and cholesterol (compound **26**, Fig. 9), synthesized by Li et al., has been tested on adenocarcinoma A549 cells, exhibiting cytotoxic activity. This action seems to be related to the inhibition of the calcium uptake and ATP hydrolysis. In addition it is possible that this compound intercalates into membranes, taking the place of physiological cholesterol, and causes modifications in the physical state of phospholipids with a consequent damage of the whole system [93]. With the same principle a molecule containing a fullerene core and a 4-aryl-1,4-dihydropyridine (DHP) has been prepared (compound **27**). This latter group is one of the most common calcium channels modulator drugs [94].

The synthesis of many fullerene derivatives bearing sugar molecules has been explored, as D-mannosyl-fullerols [95]. These derivatives can avoid the erythrocyte aggregation, typical for fullerols. The presence of α -D-mannosyl portion reduces significantly this action and also the binding to β -galactoside specific protein. In

particular for derivative **28**, aggregation does not appear even at concentrations $> 10^{-2}$ M.

Recently the 1,2-(dimethoxymethano)fullerene has been demonstrated to inhibit amyloid peptide aggregation by binding to the central hydrophobic motif of the peptide. This application places fullerene in the category of really promising Alzheimer's disease therapeutics [96].

Fullerenes and related structures have an important role also the preparation of biomaterials. Recent work has been performed in the modification of lipid membranes to obtain sensitive element to be applied in the preparation of sensors for odorant, taking advantage of fullerene electron mediator properties [97]. It has been used also to study cell adhesion and cell growth on polyurethane surface immobilized with C_{60} [28].

11. Conclusion and perspectives

Many steps forward have been performed since the very first biological experiments concerning C_{60} or other fullerenes. The intrinsic hydrophobicity has been overcome using perfectly water-soluble fullerene derivatives. The research in this field is very active and new breakthroughs are expected at any time. In fact, the unique structure and properties of fullerenes are unequalled in nature, so that their exploitation in biological application deserves a broad testing.

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