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FULLERENES FOR BIOMEDICAL APPLICATIONS

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Abstract

Fullerene (C_{60}), a carbon buckyball, was discovered by Harold Kroto, James R. Heath, Sean O'Brien, Robert Curl, and Richard Smalley in 1985 and fullerene (C60) was prepared in large amounts by Krätschmer and Huffman in 1990. Research on fullerenes has been taken actively. Fullerenes have been exploited in electronics as well as in medicine. To use the fullerenes for biological applications the problem of its solubility in water has been overcome by making their derivatives. Here , in we describe the antiviral activity, antioxidant activity, use of fullerenes in drug and gene delivery. Trans isomer of dendrofullerene shows the highest anti-HIV-protease activity. The antioxidant properties of fullerenes due the presence of large amount of conjugated double bonds and low lying lowest unoccupied molecular orbital have made fullerenes the world's most efficient radical scavenger , called radical sponges . DNA-functionalized fullerenes have been used in gene delivery as they are able to enter the cells and show comparable or even better efficiency than that of commercially available lipid-based vectors. The fullerenes act as a photosensitizes. Their applications in photo cleavage and photodynamic therapy & anticancer photodynamic therapy have been described. Dimalonic derivative (DMA C60), is a very promising agent in photodynamic therapy as it is found to exerts phototoxic effect by damaging mitochondrial membrane in HeLa cells on short-time irradiation and low power. The antibacterial activity, applications of endohedrals fullerenes in MRI and applications of fullerenes in osteoporosis are also described. The use of functionalized fullerenes such as polyhydroxyfullerenes in photoacountic imaging , photothermal treatment , antitumore treatment is explained. Use of fullerene liposome for chemotherapy and cancer theranotes is described. Application of fullerenes in medical therapeutics such as allergy asthma and arthritis and its immunological activity is described. The cationic fullerenes such as N,N-dimethyl bis-fulleropyrrolidium salt series and related compounds are fund to be active against *Enterococcus faecalis* (Gram positive), *E. coli* (Gram negative) and *Mycobacteria*. Polyfluoro biphosphonated fullerene derivatives are being developed as bimodal drug for osteoporosis therapy. Application of radionuclides encapsulated in fullerenes is described.The lutetium nitrides–based endohedrals metallofullerenes, pure Lutetium Nitride, namely Lu3N@C80, could find application as X-ray contrast agent, due to the large cross-section of lutetium.

Key Words: Fullerenes, HIV protease, gene delivery, radical scavenger, photodynamic therapy, endohedrals , photoacountic imaging , photothermal treatment, osteoporosis, radionuclides encapsulated fullerenes.

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Introduction

Fullerene (C_{60}), a carbon buckyball, was discovered by Harold Kroto, James R. Heath, Sean O'Brien, Robert Curl, and Richard Smalley in 1985 (Kroto *et al.,* 1985) and Fullerene(C60) was isolated from vaporized graphite by Krätschmer and Huffman in 1990(Krätschmer et.al., 1900 2) . The fullerenes was found to have a soccer-ball-like shape(Krätschmer *et al.,* 1900). Early functionalization studies of fullerene(C60) revealed that this carbon allotrope can undergo a variety of chemical reactions characteristic of electron deficient polyolefins (Taylor *et al.,* 1993). Fullerene(C60) consists of twelve pentagonal rings that are isolated by twenty hexagonal rings. There are [6,6] and [5,6] double bonds. The [6,6] bonds have greater double bond character and are shorter than [5,6] bonds. The [6,6] bonds are thus used to functionalize fullerene(C60) by nucleophilic, radical additions, as well as cycloadditions. The report that fullerene (C60) derivatives have biological activity, such as HIV-protease inhibition (Friedman *et al.,* 1993), photodriven DNA cleavage(Tokuyama *et al.,* 1993), radical scavenge(Dugan *et al.,* 1997), leads to the creation of a rapidly expanding new area of fullerene science(Nakamura *et al.,* 2003). Fullerenes are highly hydrophobic however, water-solubility can be achieved by attaching polar functional groups onto the fullerene structure. It has been found that stereochemically defined three-dimensional multifunctionalized fullerene structures are particularly important for biological applications, such as molecular recognition (Isaacs *et al.,* 1994). Fullerenes are inert, hollow and indefinitely modifiable. When administered orally in the water-soluble form, they are not absorbed; while on *inter vinous* injection, they get rapidly distributed to various body tissues. They are excreted unchanged by kidney (Wilson *et al.,*.). Acute toxicity of water-soluble fullerenes was found to be quite low(Yamago *et al.,* 1995).All these interesting properties offer possibilities of bright future utilizing fullerenes in biology and medicinal chemistry as medicinal agents. However, this possibility faces a significant problem, i.e., natural repulsion of fullerenes to water. To overcome this limitation, number of methodologies are being developed. These problems can be overcome by synthesizing fullerene derivatives having modified solubility profile, encapsulation of fullerene(C60) in cyclodextrins (Fillipone *et al.,* 2002) or in calixarenes (Shinka**i** *et al.,* 1999) or water suspension preparations(Scrivens *et al.,* 1994). This report reviews following important biological applications of fullerenes.

Antiviral activity : Compounds with antiviral activity are generally of great medical interest. Replication of the human immunodeficiency virus (HIV) can be suppressed by several antiviral compounds. Fullerenes (C60) and their derivatives have potential antiviral activity. It has been shown that fullerenes derivatives can inhibit and make complex with HIV protease (HIV-P) (Simon ***et al.,*** 1993 and Sijbesma ***et al.,*** 1993). Dendrofullerene , **1** (Figure 1) has shown the highest anti-protease activity (Brettreich ***et al.,*** 1998 and Schuster ***et al.,*** 2000) and compound **2** in its trans-2 isomer form (Figure 1) is a strong inhibitor of HIV-1 replication. This shows that relative position (trans-2) of substituent's on fullerenes and positive charges near to fullerenes cage is required for an antiviral structural activity.

Fullerenes C60 derivatives with two or more solubilizing side chains, are found to be active, when tested in lymphocyte CEM cell cultures infected with HIV-1 and HIV-2 (Bosi *et al.,* 2003). Amino acid derivatives of fullerene C60 (ADF) rendered water soluble by attachment of amino acids to C60:C60-l-Ala, C60-l-Ser, C60-l-Gly, C60-l-Arg and C60-d-Arg, C60-ACA (C60-ε-aminocaproic acid), C60-ACNa (sodium salt of C60-ACA), C60-ABA (C60-γ-aminobutyric

acid) and C60-ABNa (sodium salt of C60-ABA) (Romanova *et al.*, 1994, Kaesermann *et al.*, 1997 and Rywkin *et al.*, 1995). Cationic, anionic and amino acid type fullerene derivatives inhibit HIV-reverse transcriptase and hepatitis C virus replication (Mashino *et al.*, 2005). The amino acid type derivatives are found to be the most active out of all the derivatives of fullerenes.

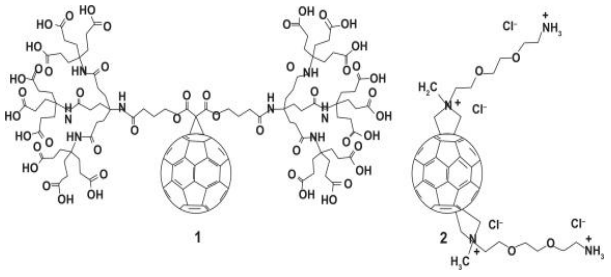


Figure- 1 Structures of compounds **1** and **2** (Brettreich *et al.*, 1998)

Fulleropyrrolidines (compound **3-7**, Figure-2) with two ammonium groups , employed in lymphocyte CEM cell cultures infected with HIV-1 or HIV-2 inhibited the antiviral activity (Marchesan *et al.*, 2005) . The results also revealed that trans fullerenes derivatives are more active than cis- counter parts whereas the equatorial one is totally inactive.

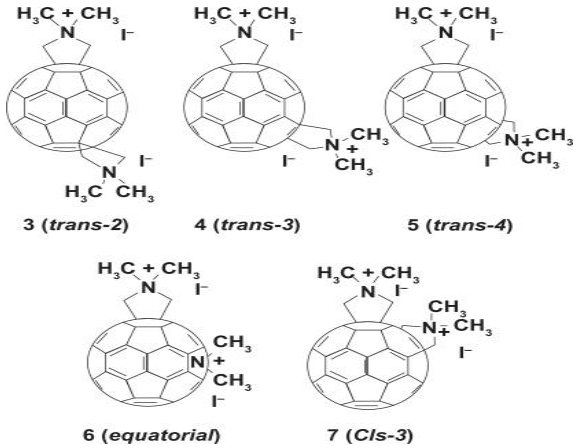


Figure- 2 Structures of Fulleropyrrolidine (Marchesan *et al.*, 2005)

The HIV-protease, is a homo-dimeric enzyme, belongs to the class of aspartic proteases (Zhu *et al.*, 2003). The hydrophobic interactions between C60 and cavity regions of enzyme hold the inhibitor tightly. This result cause the release of water, which provides indirect evidence for inhibitor presence. The synthesis and characterization of fullerene derivatives as inhibitors of HIV aspartic protease enzyme holds great potential for the development of novel anti HIV drug (Marcorin *et al.*, 2000 and Bakry *et al.*, 2007). The active region of HIV protease is a cylindrical hydrophobic cavity (diameter ~10 Å), which contains two amino acid residues, aspartate 25 and aspartate 125, whose binding causes suppression of protein slicing and inhibits viral replication. The mode of action is based on electrostatic and/or hydrogen bond interactions of fullerenes derivative side chains with Asp 25 and Asp 125, as shown in Figure- 3.

The highly water-soluble derivatives $C_{70}[p\text{-}C_6H_4(CH_2)_nCOOH]_8$ (n = 2,3) prepared from readily available chlorinated (Kornev *et al.*, 2011) fullerene precursors $C_{70}Cl_8$ and $C_{70}Cl_{10}$ has shown pronounced antiviral activity *in vitro*, particularly against human immunodeficiency virus (HIV) and influenza A virus (subtypes H1N1 and H3N2) . Recently water soluble, dendritic molecules, glycofullerenes with 12 to 36 sugar moieties on the periphery of C6 (Martín *et al.*, 2013, Sánchez-Navarro *et al.*, 2011 and Luczkowiak *et al.*, 2013) (Figure-4) have shown antiviral activity in an Ebola pseudotyped infection model in the low micromolar range for fullerenes with 12 mannoses. Interestingly, the increase of valency in glycofullerenes induced a loss of antiviral effect.

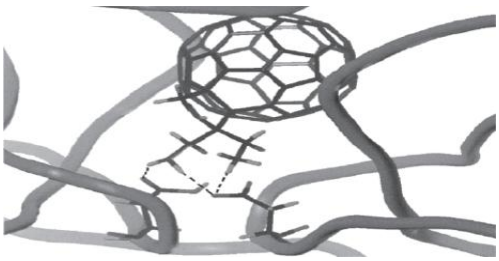


Figure-3: Closer view of the (HIV-Protease)-2a complex, showing the H-bond between NH_2 or NH^{3+} groups with Asp 25 and 125, (Marcorin *et al.*, 2000).

This could be probably related to steric congestion of sugars at the surface of the fullerene. Using a glycodendrofullerene showing the same valency but including a longer spacer, the inhibitory activity of these compounds was increased remarkebly with IC_{50} in the nanomolar range. This result highlights the importance of combining an adequate scaffold to achieve the multivalency (the spherical fullerene) with the right ligand accessibility and flexibility. Based on these results, fullerenes is considered as very attractive scaffold for a globular multivalent presentation of sugars. These promising results pave the way to new glycodendrofullerenes endowed with other biologically active molecules in the search for new applications.

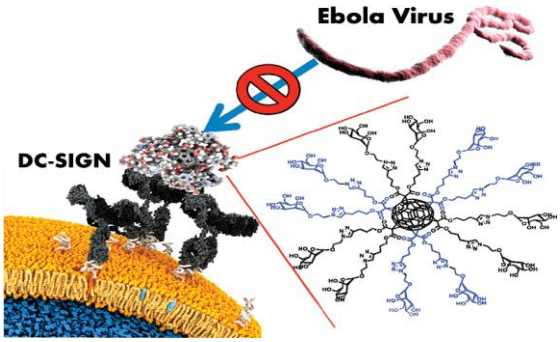


Figure- 4 : Ebola Virus glycoconjugate interaction with fullerene derivative (Luczkowiak *et al.*, 2013)

In 1918, an influenza A pandemic caused 50 million deaths worldwide (Jeffery *et al.*, 2005) , and the development of strategies to prevent future expansions of this virus continues to be an important endeavor (Horimoto *et al.*, 2005). The avian H5N1 influenza A virus is highly pathogenic to humans(Hatta *et al.*, 2001), and the emergence of a new strain of this virus in 2009, the swine-originating A/H1N1 pdm influenza virus, further emphasizes that this issue is a serious global health problem (Itoh *et al.*, 2009 and Neumann et al., 2009). The inhibitors of influenza A, e.g., the neuraminidase-like compound oseltamivir, show (De Clercq *et al.*, 2006 and Frederick *et al.*, 1999) adverse effects and resistant to these drugs have now been reported (Patrick *et al.*, 2008 and Reece *et al.*, 2007) The influenza A genome consists of segmented single-stranded RNA (-). Its transcription and replication require the activity of a highly conserved RNA-dependent RNA polymerase (Honda *et al.*, 1997 and Honda *et al.*, 2002). This polymerase is essential for the propagation of the influenza A virus and is a very promising target for the development of antiviral drugs. The influenza A virus RNA polymerase is composed of three subunits–PA, PB1, and PB2 (Kuzuhara *et al.*, 2003) – and synthesizes viral mRNA using short capped primers that are cleaved from the host's cellular pre-mRNAs by the viral endonuclease (Dias *et al.*, 2009 and Yuan *et al.*, 2009). have shown that the N-terminal domain of the PA subunit contains the active site of the endonuclease, and that this domain also harbors RNA/DNA endonuclease activity (Dias *et al.*, 2009 and Yuan *et al.*, 2009). Hence the PA endonuclease is a very effective targets for the development of novel anti-influenza A drugs. It has been shown that several chemicals, e.g., catechins, phenethylphenyl phthalimide analogs, and marchantin analogs, inhibit this endonuclease and possess

antiviral activity (Iwai *et al.,* 2010, Iwai *et al.,* 2011 and Kuzuhara *et al.,* 2009a). Recently Shoji et. al. (Shoji *et al.,* 2013) used an *in vitro* influenza PA endonuclease assay to analyze the effects of 12 different fullerene (Figure- 5), (compound **8-19**) derivatives on the endonuclease activity of the PA N-terminal domain and full-length PA. They found that the fullerene derivatives inhibit influenza PA endonuclease activity and viral infection. Their results indicate the possibility of developing fullerene derivatives into novel anti-influenza A drugs in the future.

Antioxidant activity : The antioxidant property of fullerenes is due to large number of conjugated double bonds and low lying lowest unoccupied molecular orbital (LUMO) , which can easily take up an electron. This make an attack of radical species highly possible, up to 34 methyl radicals have been added onto a single C60 molecule. The quenching process is catalytic and fullerene can react with many superoxide without being consumed. Due to this feature , fullerenes appread to be the world's most efficient radical scavenger and are called radical sponges (Krusic *et al.,* 1991). The major advantage of fullerenes as antioxidant is their ability to localize within the cell to mitochondria and other cell compartment sites, where in diseased states, the production of free radicals takes place. It has been shown that aqueous C60 suspensions prepared without using any polar organic solvent, protect their life's against free-radical damage (Gharbi *et al.,* 2005) without any toxic effects. This has been demonstrated by intoxicating rats with CCl_4, which led to the formation of trichloromethyl radical CCl_3', and CCl_3OO• on reaction with oxygen. Trichloromethylperoxy radicals a highly

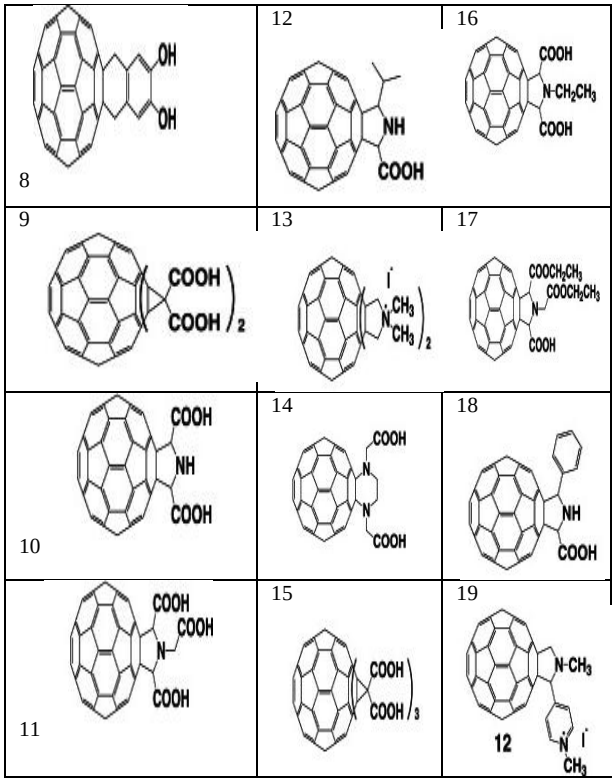


Figure-5 :12Different Fullerene Derivatives (compound **8-9,** Shoji *et al.,* 2013)

reactive species rapidly initiates the chain reaction of lipid peroxidation (Slater *et al.,* 1985). It was observed that C60 was able to scavenge a large number of these radicals per molecule leading to the result that rats pre-treated with C60 and intoxicated with CCl_4 showed no liver damage. The polar groups derivatised fullerenes such as polyhydroxylated fullerenes (fullerenol) and C60 tris(malonic)acid, cross the cell membrane and localize preferentially to mitochondria (Foley *et al.,* 2002 and Youle *et al.,* 2005) generate great masses of cellular oxygen free radicals. This phenomenon makes them useful for a variety of medical applications (Tsai *et al.,* 1997 , Lotharius *et al.,* 1997 and Bisaglia *et al.,* 2000). These radical scavengers have shown to protect cell growth from various toxins that can induce apoptotic injuries in vitro (Bisaglia *et al.,* 2000 and Lin *et al.,* 1999) in different cell types such as neuronal cells (Lin *et al.,* 1999 and Dugan *et al.,* 1997) hepatoma cells (Bisaglia *et al.,* 2000) or epithelial cells (Huang *et al.,* 1998). It has also been absorved that carboxyfullerene prevents iron-induced oxidative stress in rat brain (Lin *et al.,* 2002). The C60 tris(malonic)acid, was able to protect quiescent peripherial blood mononuclear cells against apoptosis by preserving the mitochondrial membrane potential integrity, which is the early stage of apoptosis (Straface *et al.*,1999).

The ultraviolet A radiation (320–400nm) have a biological effect on human skin cells, leading to cell damage or cell death due to the generation of reactive oxygen species such as O_2•$^-$ (superoxide), •OH (hydroxyl) radicals and closed shell H_2O_2 molecules (Monti *et.al.,* 2000). Radical Sponge® (C60 with poly(vinylpyrrolidone)) enter into depth of human skin epidermis due to its stability towards oxidative decomposition makes it more reliable than Vitamin C. Radical Sponge® enables the prevention of both UV skin-injuries and skin aging, without photosensitization and cytotoxicity (Azzam *et al.,* 2004). In cell culture experiments, C60 tris(malonic)acid rescued cortical neurons and was found to show robust neuroprotection in a number of other cell culture models of neurological disease including Parkinson's disease (Chen *et al.,* 2004). C(3)-tris-malonyl-C(60)-fullerene (Figure 6, compound **8**), while nontoxic for human keratinocytes,significantly reduced the UVB-induced inhibition of keratinocytes proliferation. It also protected keratinocytes from apoptosis caused by UVB irradiation and apoptosis induced by exposure to deoxy-D-ribose (Halliwell *et al.,* 1992 and Cheng *et al.,* 2000). The excessive production of oxygen and nitric oxide radicals is significant in the pathophysiology of Parkinson, Alzheimer, and other neurodegenerative diseases. The neuroprotective activities of carboxyfullerenes evaluated showed that regioisomers of compound 8 inhibited the excitotoxic death of cultured cortical neurons (Fumelli *et al.,* 2000). Lin et al. also showed a protective effect of compound ,20 , in the cerebellar granule cells apoptosis induced by oxidative stress (Halliwell *et al.,* 1992). Regarding the neuroprotective activity of carboxyfulleres in cells Sitharaman et al. evaluated the behavior of C3 (e,e,e tris-malonic acid-C60) in water (Sitharaman *et al.,* 2004). They found that C3 aggregates in water and that the aggregates of 40-80 nm, but not an individual molecule, are responsible for their neuroprotective action in cells (Lin *et al.,* 2002).

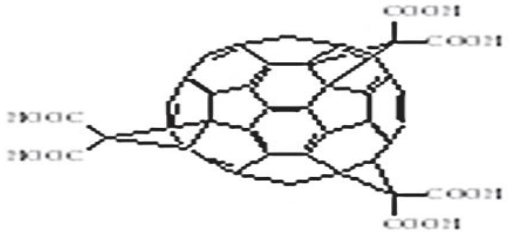


Figure 6 Structure of (3)-tris-malonyl-C(60)-fullerene (compound) **20** (Sitharaman *et al.,* 2004)

It has been shown in the past (Xiao *et al.,* 2002) PEG-modified fullerene, hydroxy-fullerene and isostearate-mixed fullerene scavenged chemically generated hydroxyl radicals as efficiently as ascorbic acid (Asc) or its 2-O-phosphorylated derivative (A2P) as shown by the DMPO-spin trap/ESR method. Enzymatically generated superoxide anion radicals were also scavenged by PEG-modified or PVP-entrapped fullerene similarly as done by Asc or A2P (Xue *et al.,* 2005 and Xiao *et al.,* 2005).

Zhou *et al*. (Zhiguo *et al.,* 2013) has reported the design and synthesis of a new class of amphiphilic fullerenes, their liposome formulation and biological activities as free radical scavengers (Rossi *et al.,* 2013). Figure 7 illustrates the design strategy in which the amphiphilic fullerenes and phospholipids co-assemble bilayer vesicles. This method leads to highly increased loading capacity of fullerenes. The amphiphilic fullerene form bilayer vesicles with lipid-to-fullerene molar ratio greater than 1:1 to produce uniform and dimensionally stable vesicles. They also reported that the oval structure of C70 molecule (as opposed to the spherical C60) provides a novel structural platform to prepare this new type of amphiphilic fullerenes. C70 has two reactive

poles and a relatively inert equatorial region, and this allows for sequentially attaching lipophilic and hydrophilic groups at the two poles, respectively. The large un-derivatized zone around the C70 belt has very high radical reactivity due to the significant orbital overlap of its lowest unoccupied molecular orbital (LUMO) and highest occupied molecular orbital (HOMO) that is expected for sites of maximum radical reactivity (Gan *et al.,* 2002, Birkett *et al.,* 1995 and Scuseria *et al.,* 1991). The fullerene-enriched liposome provides a novel formulation approach that not only enhances the fullerene delivery efficiency, but also maintains their antioxidative properties.

By mimicking the structure of naturally occurring lipid molecules, this design overcomes several limitations in fullerene liposome formulations including (i) low fullerene content when un-derivatized fullerenes are used and (ii) damaged antioxidative bioactivity due to the additions of multiple groups to fullerenes. The strong association between amphiphilic fullerenes and auxiliary lipids allow them to form dimensionally stable liposomes with as high as 65% (by weight) fullerene. The antioxidant property of fullerenes is retained in the bipolarly functionalized C70 derivative, amphiphilic liposomal malonylfullerene (ALM), as well as in its liposomal formulations, as shown by both electron paramagnetic resonance (EPR) studies and *in vitro* reactive oxygen species inhibition experiments. The liposomally formulated ALM efficiently quenched hydroxyl radicals and superoxide radicals. In addition, the fullerene liposome inhibited radical-induced lipid peroxidation and maintained the integrity of the lipid bilayer structure. This new class of liposomally formulated, amphipathic fullerene compounds represents a novel drug delivery system for fullerenes and provides a promising pathway to treat oxidative stress-related diseases.

In order to increase the efficiency of delivery of fullerenes to target tissues, lipophilic fulleropyrrolidine derivatives Q-C60: [*N*-methyl-(2-quinolyl)fulleropyrrolidine] and I-C60:[*N*-methyl-(2-indolyl)fulleropyrrolidine] were also synthesized and encapsulated in multilamellar phospholipid liposomes, and the antioxidative capacity was studied using

EPR spin-trapping and spin-labeling techniques (Lens *et al.,* 2008). Its capacity for removal of •OH (hydroxyl radical) and O2 •−

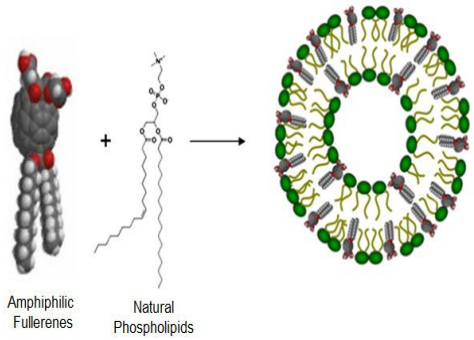


Figure 7. Vesicle formation of amphiphilic fullerene compound ALM with auxiliary phospholipids.

(superoxideradical) and for the prevention of lipid peroxidation were compared with the performance of pristine C60, Q-C60 and I-C60 showed similar, or even better, antioxidative characteristics. Other fulleropyrrolidine derivatives were also reported for incorporation in liposomes (Williams, *et al.,* 1996).

Fullerenes in drug and gene delivery : Transport of any compound into the nucleus of an intact cell is restricted by three membrane barriers which are the cell membrane, the endosomal membrane and the nuclear membrane. Hence before deciding a drug and gene carrier it is important to fully understand the mechanism through which carriers enter the cells. Four major groups of drug and gene carriers are , organic cationic compounds, viral carriers, recombinant proteins and inorganic nanoparticles (Lin *et al.,* 2002 and and Zhi *et al.,* 2005). A large number of nanoparticles have been tried as carriers for the cellular delivery because of their versatile properties, including good biocompatibility, selective targeted delivery and controlled release of carried drugs. Fullerenes are inorganic nanoparticles with small size (~ 1 nm) and biological activity. The activities of this allotropic form of carbon rest upon the properties of both, the hydrophobic fullerene core and functional groups attached to the core. By attaching hydrophilic moieties, fullerenes become water-soluble. The water soluble derivatized fullerene can cross the cell membrane and bind to the mitochondria (Williams *et al.,* 1996).

It has been reported that DNA-functionalized fullerenes are able to enter the COS-1 cells and show comparable or even better efficiency than that of commercially available lipid-based vectors (Nakamura *et al.,* 2003) *{COS is a fibroblast-like cell line derived from monkey kidney tissue. COS cells are obtained by immortalizing CV-1 cells with a version of the SV40 virus that can produce large T antigen but has a defect in genomic replication.]* Biochemical studies on the mechanism of transfection indicate that the fullerene reagent increases the lifetime of DNA in endosomes and thus supports their chromosomal incorporation by forming a protective sheath around bound DNA (Isobe *et al.,* 2006). The lipophilic slow-release drug delivery systems which employ fullerene derivatives to enhance therapeutic efficacy in tissue culture have also been studied (Tatiana *et al.,* 2005 and Ryman-Rasmussen *et al.,* 2006). The ability of fullerenes to penetrate through intact skin is widening their application in cellular drug and gene delivery (Rasmussen *et al.,* 2006). A fullerene-based peptide has shown its ability to penetrate through flexed and unflexed skin (Rouse et al., 2007). Studies carried out on the biological efficacy of water-soluble fullerenes *in vitro* (Tsuchiya *et al.,* 1995 and Lin *et al.,* 1999) and *in vivo* (Yamago *et al.,* 1995 Sato *et al.,* 1997) indicated low toxicity. The fullerenes also did not have the ability to induce acute oral toxicity or *in vitro* genotoxicity (Takada *et al.,* 2006). Although water-soluble fullerenes are not acutely toxic, they are retained in the organism for long periods, which is raising concerns about chronic toxic effects (Sato *et al.,* 1997). However , there is striking evidence that hydrophilic functional groups on the surface of fullerenes dramatically decrease toxicity of raw C60 molecule (Sayes *et al.,* 2004). Underivatized fullerenes aggregate in water are supposed to cause oxidative damage to cellular membranes even at relatively low concentrations (20 ppb level). In an effort to elucidate the relationship between the hydrophobicity of the fullerene core, the hydrophilicity of the water-solubilizing groups, and the overall charge state of the C60 vectors in gene delivery and expression, several different C60 derivatives were synthesized to yield either positively charged, negatively charged, or neutral chemical functionalities under physiological conditions (Sitharaman *et al.,* 2008).

Recently a DNA delivery system has been studied using fullerenes (Maeda-Mamiyaa *et al.,* 2010), that have DNA-binding side chains. Cationic fullerene molecules such as tetraaminofullerene are capable of condensing double strand DNA into globules smaller than 100 nm. They can therefore penetrable into the cell (Isobe *et al.,* 2006 and Isobe *et al.,* 2006). The more hydrophobic nature of fullerenes than alkyl chains in lipids appears to enable fullerenes to form a stable complex with DNA. Tetraaminofullerene was producible in only two steps with fullerene and piperazine derivatives. Moreover, a cationic fullerene can induce gene expression by releasing DNA inside the cell because of its resistance to digestion by nucleases. Earlier, the synthesis of tetra(piperazino)fullerene epoxide (TPFE) (Figure 8 ,compound, **21**)) and its binding abilities to DNA, where protection against endonuclease by TPFE was confirmed (Isobe *et al.,* 2006). In vitro gene delivery efficiency was examined by adjusting various conditions including the fullerene/base pair ratio, the transfection time, and the amount of plasmid DNA. TPFE showed a 4-fold increase of transfection efficiency compared to Lipofectin. Furthermore, TPFE showed reduced cytotoxicity, indicating that TPFE can be used for in vivo transfection (Song *et al.,* 1997 and Hofland *et al.,* 1997). *To date, no report in the literature has described in vivo gene delivery using carbon nanomaterials*. Because TPFE-DNA complex completely differs from Lipofectin-DNA complex in terms of size, charge, and conformational structure, it is expected that gene delivery by TPFE will have different organ affinity and might be able to overcome the problem of being trapped by the lung in the first pass (Thierry *et al.,* 1997). This study was undertaken to develop an *in vivo* gene transfer system using a cationic fullurene TPFE. The efficacy of gene delivery to the fetus, organ safety issues, and therapeutic application by TPFE were also evaluated.

Figure-8 : Tetra(piperazino)fullerene epoxide (TPFE), (compound **,21**).

In conclusion, it is demonstrated that a water-soluble fullerene TPFE can deliver genes as efficiently as a cationic liposome based system in vivo. In fact, TPFE can produce a stable complex with DNA and show no acute organ toxicity. Moreover, insulin gene delivery by TPFE could reduce blood glucose levels. Recently polyplexes prepared from DNA and globular compact polycationic derivatives constructed around a fullerene hexakis-adduct core have also been shown remarkable gene delivery capabilities (Sigwalt *et al.*, 2011).

Photocleavage and Photodynamic Therapy : Irradiation of fullerene with UV and visible light yields the excited singlet state, which decays to a long-lived triplet state. However, the excited state can be quenched by oxygen before a spontaneous relaxation. Therefore fullerene can acts as a photosensitizer (Orlova1 *et al.,* 2013). It can use the light energy to produce reactive oxygen species, with a very high efficiency. This phototoxic effect can be exploited for therapeutic uses. It is possible to administrate a harmless compound (a photosensitizer) and locally trigger the toxicity by focused irradiation, as in photodynamic therapy. Hence it is possible to selectively destroy tumor cells, viruses or bacteria. Fullerene seems to be the ideal scaffold for the design of novel photosensitizers, due to its low toxicity in the dark and the high quantum yield (number of $^1O_2$ produced per absorbed photon), near to unity.;

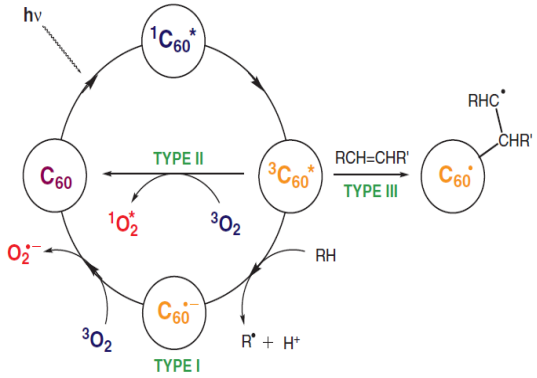


Figure 9: Schematization of the three possible quenching pathways the fullerene triplet state can undergo, exerting its photodynamic activity (Orlova1 *et al.,* 2013).

There are different pathways for quenching of the $^3C_{60*}$ is shown in Figure 9.

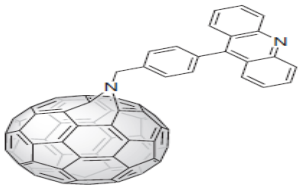
(i) It can directly transfer the energy to the molecular oxygen yielding highly reactive $^1O_2$ (energy transfer or Type II) in organic solvents such as benzene and toluene.

(ii) Otherwise, because the triplet state of fullerene is an excellent electron acceptor, it can be easily reduced to radical anion (charge transfer or Type I) in more polar environments and in presence of reducing agent such as NADH. However, the two mechanisms can be present at the same time.

(iii) Another possibility, although uncommon, is the direct reaction of $^3C_{60*}$ with biomolecules, destroying the photosensitizer and the biomolecules at the same time (Type III) (Markovic *et al.,* 2008 and Mroz *et al.,* 2008).

a.DNA photocleavage : The direct interaction between photoactivated C60 and DNA can lead to the oxidation of guanine to 8-oxoguanine, hence the weakened bond is hydrolyzed resulting in the cleavage of DNA strand (Bernstein *et al.,* 1999) Water soluble γ-cyclodextrin-bicapped C60 has shown a DNA photocleavage activity only in presence of NADH, suggesting a type I mechanism ($O2^{\cdot-}$) and the activity was found to be dose dependent from both γ-CD-C60 and NADH (Nakanishi *et al.,* 2002). A similar activity was realized with a calixarene analog, functionalized with several trimethylammonium moieties (Ikeda *et al.,* 1999). Unmodified C60 was tested also when incorporated in liposomes. Liposomes were loaded with a high concentration of fullerene through exchange with γ-CD-C60 complex. Cationic liposomes were effective in DNA photocleavage, while anionic liposomes, as expected, did not show activity because of the electrostatic repulsion with DNA (Ikeda *et al.,* 2005). A similar study was conducted with C70 showing that cationic liposome-C70 has a 3.5-fold higher DNA photocleavage activity than cationic liposome-C60 (92% *vs.* 26%) under the same experimental conditions (2 h, visible light) (Ikeda et al., 2007). It has been observed an extensive functionalization of fullerenes breaks the π-system, which results in a decrease of the photoexcitability. A study on six different fullerene polyadducts has clearly demonstrated that effectiveness as photosensitizer is inversely proportional to the degree of functionalization (Mroz *et al.,* 2007).

In order to increase specificity toward DNA and/or particular regions of the double strand. the acridine-C60 adduct, **22** (Figure 11) has been prepared. With this acridine- C60 adduct, acridine, intercalating the DNA, drives the fullerene in proximity of the double strand. The adduct, solubilized in PVP, showed a good photocleavage activity, much higher than pristine fullerene (90% *vs.* 26%) in the same conditions (1 h, visible light) demonstrating the effectiveness of the acridine–DNA interaction. In order to achieve higher selectivity for DNA and for some specific sequences, derivatives containing a minor groove binder (MGB) and an oligonucleotide were synthesized such as compound **23**, (Figure 10) (Bergamin *et al.,* 2011, Da Ros *et al.,* 2002 and Da Ros *et al.,* 2002a). The introduction of a long chain between the MGB and the fullerene core leads to a good interaction with DNA, as demonstrated by an enhanced photocleavage activity of **24**, in comparison to the analogous without the MGB.



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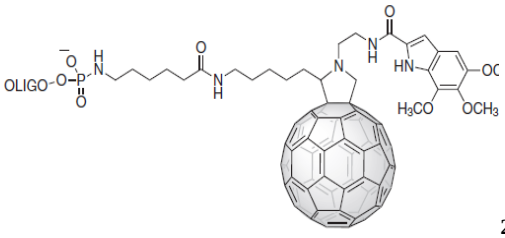

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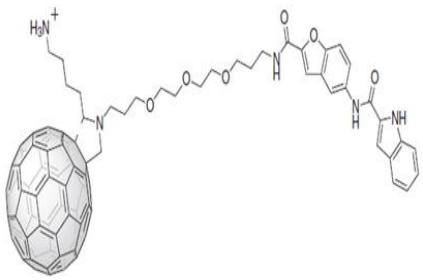


24

Figure 10. Structures of fullerene derivatives able to interact with DNA: acridine–fullerene derivative **22**, and two examples of minor groove binder-fullerene conjugates **23** and **24.**

Efficient photocleavage of DNA utilising water-soluble lipid membrane-incorporated [60]fullerenes has been reported using a [60]fullerene exchange method (Ikeda *et al.,* 2005a). Efficient photocleavage of DNA utilizing water soluble riboflavin/naphthaleneacetate substituted fullerene complex has also been recently reported (Gao *et al.,* 2009). An extremely effective DNA photocleavage utilizing functionalized liposomes with a fullerene-enriched lipid bilayer (Ikeda *et al.,* 2007a) has been developed. Efficient photocleavage of DNA utilising water-soluble lipid membrane-incorporated [60]fullerenes prepared using a [60]fullerene exchange method (Ikeda *et al.,* 2005b) was reported recently. Recent studies showed that a super-highly hydroxylated fullerene derivative protects human keratinocytes from UV-induced cell injuries together with the decreases in intracellular reactive oxygen species (ROS) generation and DNA damages (Saitoh *et al.,* 2011).

b.Anticancer photodynamic therapy : The first experiment of photodynamic cell inactivation using fullerene was reported in 1993 (Tokuyama *et al.,* 1993). Water-soluble monoadduct **25** (Figure 11), togetherwith its triethylammonium salt, was found to be cytotoxic against HeLa S3 cell line under visible light, while in the dark it did not show any cytotoxicity. These findings demonstrated for the first time the feasibility of photodynamic therapy (PDT) with fullerenes derivatives. A good dispersibility of the C60 derivatives is an essential requirement for fullerene-based PDT because of the shortening of the excited triplet state lifetime within the aggregates, meaning a loss of photodynamic activity for sparingly soluble derivatives. A study compared the photodynamic activity of C60(OH)24 and γ -CD-C60 complex in HaCaT cells. It was found thet fullerenol has a much higher cellular uptake than the γ -CD-C60 complex cytotoxic but cyclodextrin complex, was found to be 60 times less cytotoxic (Zhao *et al.,* 2008). Liposomes encapsulated (Mroz *et al.,* 2007) C60 and C70 in HeLa cells cultures, and almost all the cells underwent apoptosis after irradiation .Exchange reaction between γ -CD-C*x* (*x*=60, 70) and HeLa cells was performed at 37ºC .(Ikeda *et al.,* 2007b and Doi *et al.*,2008).

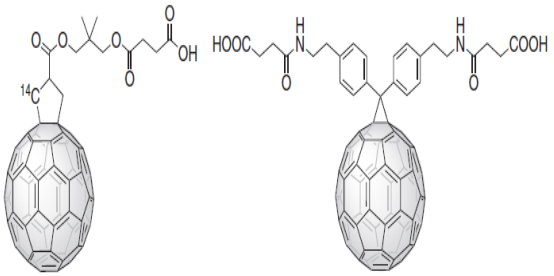
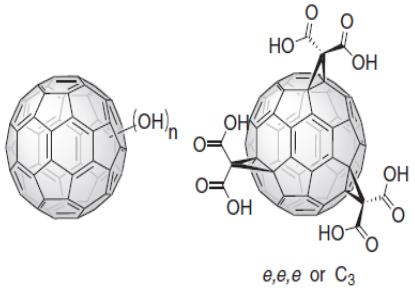


Figure-11 , Water-soluble monoadduct, **25** together with its triethylammonium salt.

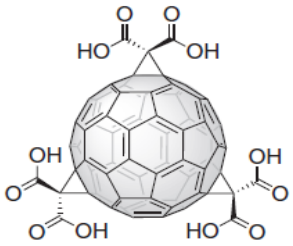
Exchange reaction with a good uptake of C70, while a minor uptake of C60 was observed in these conditions, due to the greater stability of γ -CD-C60. After a 30-min photoirradiation, only the 25% of the γ -CD-C70 treated cells survived, while most of the cells survived after γ-CD-C60 photoirradiation, signifying a very high effect of C70 on cell membranes (Ikeda *et al.,* 2009).

Dendrofullerene 1 (Figure 1) and C3 derivative (compound **27**, Figure 12) are probably the most studied water-soluble fullerenes. Their photodynamic properties are also evaluated in detailed. The dendrofullerenes reversibly inhibited proliferation in two-week cultivation of Jurkat cells while C3 has only little effect; after irradiation with UVA and UVB light. Both the fullerenes have a toxic action, but photocytotoxicity of C3 is more pronounced.



e,e,e or C_3

26 **27**



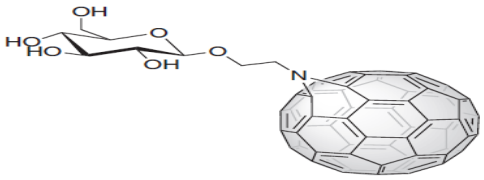
trans-3,trans-3, trans-3 or D_3

28

Figure-12 : Structure- of fullerenenol **26** , derivative **27** , C3 and **28** ,

Dimalonic derivative (DMA C60), seems to be very promising agent in PDT as it is found to exerts phototoxic effect by damaging mitochondrial membrane in HeLa cells, (Yang et.al., 2007) on short-time irradiation and low power. A PEG-C60 when retained in tumors for a long period, and after photoirradiation, all the tumor-bearing mice were cured (Tabata *et al.,* 1997).

Rapidly proliferating cells, such as cancer cells, have enhanced uptake rate of nutrients, such as glucose, amino acids and folate. It is known that folic acid receptor is very common tumor marker, over expressed in several kinds of



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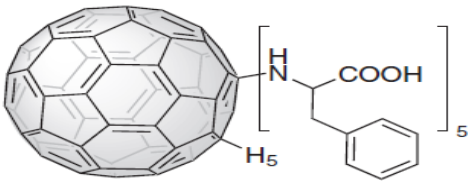

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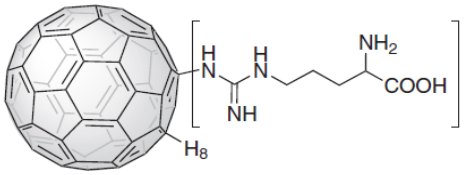

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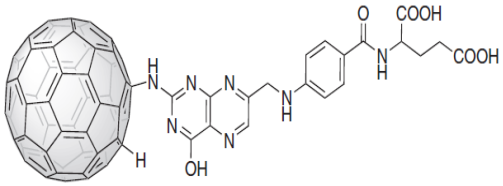


32

Figure 13. Chemical structures of glucose derivative **29**, two amino acid derivatives of fullerene (**30** and **31**) and of folate derivative **32**.

cancer. Among some glycoconjugates synthesized , the derivative **29** presenting one glucose residue (Figure 13) was the most effective *in vitro* against several types of cancer, and it was assayed also *in vivo*. Melanoma (COLO679) was photodynamically suppressed in mice after repeated PDT in presence of **29** (Otake *et al.,* 2010). Recently, the synthesis and the photodynamic activity of amino acid fullerene compounds (**30** and **31**, Figure 13) and folate fullerene derivative (**29**,Figure 13) have been reported. All these molecules are more efficiently internalized in HeLa cells than in N2a cells, indicating selectivity for cancer, with the best targeting activity and internalization for **32**, which results also the most cytotoxic upon photoirradiation. Moreover, an increase of caspase-3 activity was observed with subsequent apoptotic cell death. For these reasons, bioconjugates **30–31** are very promising photosensitizers in cancer therapy (Hu *et al.,* 2010).

Conventional photodynamic therapy nowadays is based on tetrapyrroles, such as porphyrins, chlorins and pyropheophorbides. Porphyrin-C60 dyads,when irradiated, give rise to a long-lived radical ion pair ($P^{\cdot+}$−$C60^{\cdot-}$) through an electron transfer reaction. A dyad containing three methoxy phenyl groups on the porphyrin stabilize the positive charge on the tetrapyrrole (Figure 14). Due to its insolubility, compound ,**32** was administrated as liposomial inclusion. After incubation in the dark, no cytotoxic effect was observed, while, after irradiation, up to the 80% of cells were inactivated and this compound is able to kill cells even in hypoxic environment (Alvarez *et al.,* 2006).

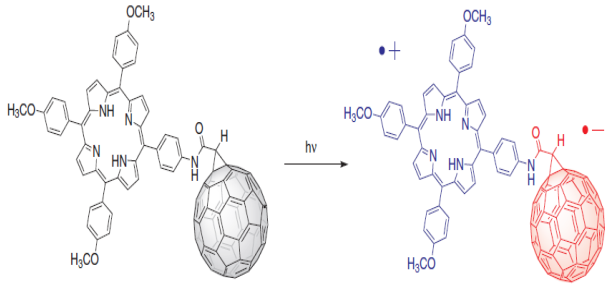


Figure 14. Structure of dyad **32** and its charge separated state after photoirradiation

Fullerene was used as a drug delivery system for pyropheophorbide: few derivatives were synthesized, bearing 2, 6 (compound **33**, Figure 15) or 12 pyropheophorbide moieties and, in a more complex molecule, an antibody (Ab) was conjugated to a fullerene with 10 units of pyropheophorbide, to enhance the tropism for some tumors, specifically recognized by the Ab.

The hexakis addition is used to disable the photochemical properties of the fullerene moiety by breaking the C60 π-system, to prevent the electron transfer process from inhibiting the production of $^1O_2$, thereby obtaining a good PDT response from the tetrapyrrole (Ermilov *et al.,* 2004 and Rancan *et al.,* 2007). Several fullerene derivatives liposome have also been used for photodynamic therapy (Yamakoshi *et al.*,, 2003 , Ikeda *et al.,* 2007c, Ikeda *et al.,* 2005c, Ikeda *et al.,* 2011 and Du *et al.,* 2012).

Antibacterial activity : Since then the observation that a simple water suspension of fullerene has an antibacterial activity (Lyon *et al.,* 2005) and can be used for disinfection of waters and soil has promoted the researchers to to study the antibacterial activity of fullerene and its derivatives. The mechanism of *n*C60 antibacterial effect is still debated but was found to be related to disruption of cell membranes. (Fang *et al.,* 2007 and Tsao *et al.,* 2002). But in some cases contradictory results were reported, confirming the membrane integrity (Aquino *et al.,* 2010 and Lyon *et al.,* 2006). *In vitro* studies on the well-known C3 carboxyfullerene (27, Figure 8) reported its antibacterial activity on Gram-positive species (Tsao et al., 2001). C3 is also active *in vivo*, in fact, it can protect mice from lethal infection of *Streptococcus pyogenes*(Mashino *et al.,* 2003).

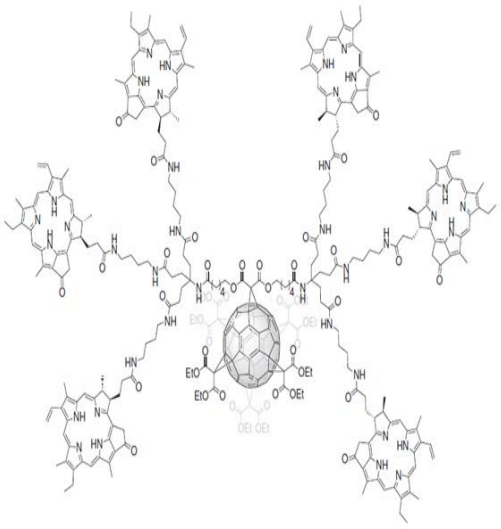
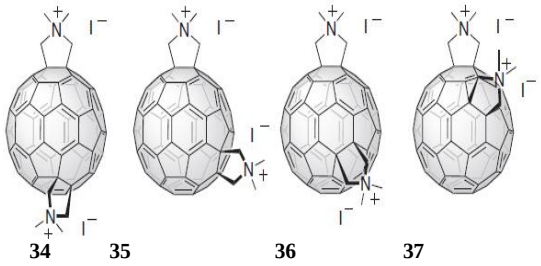


Figure-15 Hexakis adduct bearing six pyrophenorbide units (compound **33**)

Cationic fullerenes such as N,N-dimethyl bis-fulleropyrrolidium salt series (Figure 16) and related compounds are active against *Enterococcus faecalis* (Gram positive), *E. coli* (Gram negative) (Bosi *et al.,* 2000) and *Mycobacteria* (Mashino *et al.,* 1999). The mechanism action of cationic fullerenes was not clarified but in some other cases, the cationic derivatives act without disrupting bacterial wall; in fact, respiratory chain inhibition was reported (Mashino *et al.,* 2003). Among the of N,N-dimethyl bis-fulleropyrrolidium salts series *trans-2* and *trans-4* isomers (**34** and **36**, Figure12) are the most active compounds with an antibacterial activity on *E. coli* comparable to vancomicine. At low doses the uptake of oxygen is inhibited, while at high doses it is enhanced, with concomitant production of hydrogen peroxide (Tang *et al.,* 2007). The cationic fullerenes have high affinity towards negatively charged bacterial membrane and consequently show higher activity with respect to anionic or neutral fullerene compounds (Tegos *et al.,* 2005). Mono-, bis- and tris-N,N-dimethyl fulleropyrrolidium salts are able to kill more than 99.99% of bacteria when photoirradiated (Tegos *et al.,* 2005). Structural variations on cationic fullerenes gave similar results and confirmed once more the importance of multiple positive charges in addressing the compounds to bacteria (Spesia *et al.,* 2008 and Huang *et al.*,, 2010). The broad activity spectrum, including both Gram-positive and Gramnegative microorganisms, which present different membrane permeability, was explained by a self-promoted uptake of cationic molecules, counteracting the differences in permeability (George *et al.,* 2009). The broad activity spectrum, including both Gram-positive and Gramnegative microorganisms, which present different membrane permeability, was explained by a self-promoted uptake of cationic molecules, counteracting the differences in permeability (Mizuno *et al.,* 2011).



34 **35** **36** **37**

Figure 16 , N, N'-dimethyl bis –fulleropyrrolidinium regioisomer **34-37** respectively , trans 2 , trans 3, trans 4 and cis 3.

The amphiphilic nature of fulleropyrrolidine derivatives was , antimicrobial peptides (Pantarotto *et al.,* 2002) active against Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*E. coli*). But for the latter the response was less extended. A new dendrofullerene-bearing sugar unit has been demonstrated to bind bacterial lectin of *Pseudomonas aeruginosa*, an opportunistic pathogen. The lectin is involved in the recognition of glycoconjugates on human tissues, and this process could be prevented if lectin would be masked by the sugar fullerene derivative (Cecioni *et al.,* 2011). An analogous derivative was also reported to inhibit glucosidase activity (Compain *et al.,* 2010). Finally, QSAR evaluation of cationic fullerenes as antibacterial photosensitizers underlined that the effectiveness was related not only to the number of the charges but also to their positions on the sphere. A wide dispersion of the positive charges on sphere surface inhibits the photosensitizer aggregation with an associated increase of activity (Pantarotto *et al.,* 2002). Antibacterial effects for C60 (Fortner *et al.,* 2005 and Tsao *et al.,* 2002), and its hydroxylated derivatives. amino-derivatives,orcarboxyfullerenes have been investigated on bacteria *E. coli* and *B. subtilis* (Aoshima *et al.,* 2009 , Tang *et al.,* 2007, Kang *et al.,* 2009 , Lyon *et al.,* 2008 and Lyon e *et al.,* 2008a). Results of the studies indicate that the level of antimicrobial effects depends on a specific derivative of C60 fullerene, on a selected testing organism and on environmental conditions (pH, electric charge, salinity). Recently it has been reported that C60 fullerene derivatives had negative effects on the selected groups of unicellular organisms. The most noticeable negative effect was found for the bromo derivative, while the oxo derivative had the smallest effect (Kubatova , *et al.,* 2013).

Recently Delinay et.al. (Lyon *et al.,* 2006b 161) has examined how the morphology of the nC60 aggregate affects its antibacterial activity. They prepared fullerene water solution (FWS) using four different methods, using THF as a solvent (THF/nC60), sonicating C60 dissolved in toluene with water (son/nC60), stirringC60 powder in water (aq/nC60), and using a solubilizing agent (PVP/C60). They tested each preparation for antibacterial activity toward the Gram-positive bacterium *Bacillus subtilis*. They then examined fractions of the nC60 aggregates, paying particular attention to how the size and morphology of nC60 affect antibacterial activity. All four FWS displayed antibacterial activity toward *B. subtilis*, THF/nC60 had an minimum inhibitory concentration (MIC) of 0.09 (0.01 mg/L), son/nC60 had an MIC of 0.7 (0.3 mg/L), aq/nC60 had an MIC of 0.5 (0.13 mg/L), and PVP/C60 had an MIC of 0.95 (0.35 mg/L. At a 95% confidence interval, there is no statistical difference between the antibacterial activity of the son/nC60, aq/nC60, and PVP/nC60 preparations. THF/nC60 appears to have a more potent antibacterial effect than the other preparations, having an MIC one order of magnitude smaller.

The antimicrobial activities of fullerene C60 and its derivatives against 6 kinds of bacteria and 2 kinds of fungi have evaluated by Aoshima et.al (Aoshima et al., 2009). The tested samples were water-soluble fullerenes (polyvinylpyrrolidone (PVP)/C60, gamma-cyclodextrin (gamma-CD)/C60, and nano-C60) and 3 types of fullerenols (C60(OH)12, C60(OH)36.8H2O, and C60(OH)44.8H2O). The pristine C60 demonstrated no antimicrobial activity, fullerenols exhibited good antimicrobial activity against Propionibacterium acnes, Staphylococcus epidermidis, Candida albicans, and Malassezia furfur. In particular, C60(OH)44 exhibited a strong and wide-ranging antimicrobial activity comparable to that of catechin. This compound exhibits antimicrobial activity via inhibition of microbial cell growth and not via bactericidal activity.

Applications of Endohedrals fullerenes in biology : The details of endohedral fullerenes is recently reviewed by Popov et.al.(Popov *et al.,* 2013 and Chen *et al.,* 2012). The fullerenes can accommodate in their hollow core a number of atoms or ions. The compounds containing atom or ions in the hollow core are called endohedrals. Although the fullerene cage, can be opened, fill and re-closed as in a "molecular surgery",(Vougioukalakis *et al.,* 2010) but this is not the common way to produce endohedrals. The fullerene guest, usually a lanthanide or transition metal, is added to the carbon source used for the fullerene production and the metal is entrapped inside the cage at the moment of its closure. The metal is not trapped in the center of the cavity but is very close to the internal face, suggesting a tight electronic interaction between the carbon sphere and the metal. The endohedral is represented by La@C82, which is actually La^{3+} @C82−. This can be considered as super atom in which a positively charged nucleus is enclosed in negatively charged carbon sphere. The endohedrals Ca@C60 and Li@C60 are sparingly soluble in solvents such as toluene and CS_2, typically used for fullerenes, and much more soluble in polar solvents as pyridine or aniline. The endohedrals is not simply a "box" that encloses a metal, but has to be considered a different entity (Shinohara *et al.,* 2000). Endohedrals metallofullerene, can find application in biomedical fields, such as magnetic resonance imaging (MRI) and radioimmunotherapy. Applications of functionalized fullerencs in tumor theranostics have recently been reviewed by Chen et. al (Chen *et al.,* 2012).

a. MRI : In nuclear magnetic resonance, paramagnetic compounds are able to increase the relaxation rates of protons. Many lanthanide ions are paramagnetic due to their *f* orbitals, among the series Gd^{3+} (4*f* 7), with seven unpaired electrons in its half-filled 4*f* orbital, has the highest relaxation-increasing power on the nearby nuclei. For this reason, Gd^{3+} chelates (*i.e.*, Gd-DTPA) are commonly employed in MRI as "contrast agent" for the enhancing of relaxation rate of water protons (Caravan *et al.,* 1999). Even though these chelate derivatives are currently used in clinical diagnostics, there is always a residual risk of gadolinium (III) ion release from the chelate with the subsequent toxicity and the precipitation of $Gd(OH)_3$ and $GdPO_4$. The residual risk of gadolinium (III) ion release from the chelate cannot take place if the ion is entrapped into the fullerene cage. Therefore Gd@C2n, **38**, (Figure 17) is more suitable in MRI as contrast agents. The water solubility of Gd@C2n is improved by derivatization of M@C2n . In addition to the safety, water-soluble endohedral gadofullerenes have shown higher relaxivities than clinically used gadolinium chelates. Many Gd@C2n derivatives have been tried (Bolskar *et al.,* 2003, Zhang *et al.,* 2007 , Sitharaman *et al.*, 2004b, Toth *et al.,* 2005, Laus *et al.,* 2005 and Sitharaman *et al.,* 2007) but *in vivo* studies have confirmed the real applicability in MRI of $Gd@C82(OH)_{40}$ as a RES-specific MRI contrast agents using just 1/20 of the clinically employed dose of Gd-DTPA, depicted in Figure 13 (5 µmol Gd/kg versus 100 µmol Gd/kg) (Mikawa et.al., 2001 174) and of a Gd3N@C80 derivative the dosage of which is 1/50 of the Gd-DTPA clinical dose (Shu *et al.,* 2009, Zhang *et al.,* 2010 and Meng *et al.,* 2010). $Gd@C82(OH)_{22}$, can act as angiogenesis inhibitor down regulating more than 10 angiogenic factors as proved by mRNA and protein expression analysis, demonstrating an anticancer activity comparable to paclitaxel (Meng *et al.,* 2010).

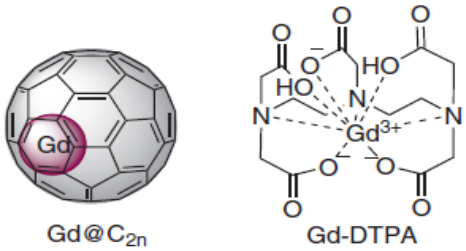


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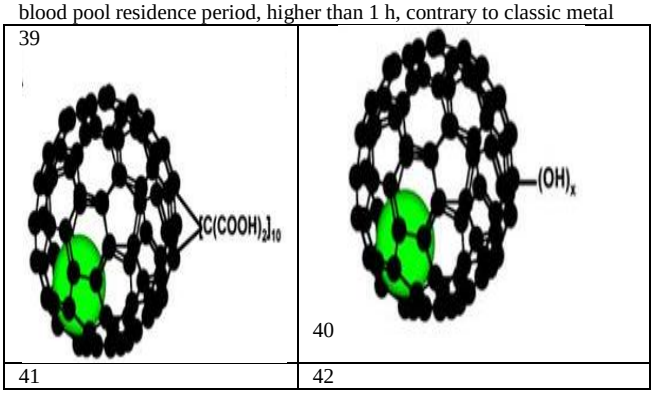
Figure-17 General structure of gadolinium endohedral fullerene (left) and Gd- DTPA complex (right) , **38** currently used as contrast agent in MRI

Number of endohedral fullerenes containing only one atom and cluster of atoms and heterogeneous atoms have been studied. Typical examples are Sc*n*@C82 (*n* = 1−4) and La2@C80 , Lu2Gd@C80 (Zhang *et al.,* 2007). In the case of the so-called GdSc2@C80–BioShuttle (GdSc2@C80 + Nuclear Localizing Sequence + Cell-Penetrating Peptide) a dramatic increase of MRI efficiency was observed in breast cancer cell cultures, up to 500-fold than commercial MRI contrast agent as Gd-DTPA. This could be probably due to the high local concentration of GdSc2@C80–BioShut (Braun *et al.,* 2010). Out of Lutetium nitrides–based endohedrals metallofullerenes, pure Lutetium Nitride, namely Lu3N@C80, could find application as X-ray contrast agent, due to the large cross-section of lutetium. But mixed nitrides such as Lu2GdN@C80, HoLu2N@C80 and Ho2LuN@C80 could be the scaffold of multimodal contrast agent for both MRI and X-ray diagnosis (Iezzi *et al.,* 2002). Paramagnetic metal ions, especially gadolinium (Gd^{3+}), have been proposed as MR contrast agents. Conventional T1 contrast agents have been developed in the form of more stable and less toxic metallochelates, including gadolinium(III) diethyltriaminepentaacetic acid (Gd(III)-DTPA) and gadolinium-tetraazacyclododecanetetraacetic acid (Gd(III)-DOTA), which are currently marketed as Omniscan® and ProHance®, respectively (Na *et al.,* 2009 and Mody *et al.,* 2009). However, for Gd^{3+} chelate complexes, the main limitation is the release of metal ions *in vivo* during metabolic processes and the subsequent toxicity. Since gadolinium-containing metallofullerenes were first used as excellent contrast agents for MRI with relatively high spatiotemporal resolution, they have attracted much attention for their potential application in clinical diagnosis. The Gd^{3+} ion was encapsulated in the fullerene cage, which is a structure that preserves the properties of the metal ion, avoids any leakage and thus prevents its dissociation *in vivo* (Mody *et al.,* 2009). After specific chemical modifications, various Gd@C_n (n=60, 82) derivatives have been developed and widely explored (Figure 18(Compound,**39**-**43**) (Sitharaman *et al.,* 2004b and Liu *et al.,* 2009).

Gd@$C_{82}(OH)_n$ has shown a water proton relaxivity twenty times higher than the commercially available Gd-DTPA. After intravenous (iv) administration of Gd@$C_{82}(OH)_{40}$ at a dose of 1/20 of the typical clinical dose of Gd-DTPA, a higher signal enhancement was measured *in vivo* (Mikawa *et al.,* 2001) To achieve better bio-distribution, modification of the fullerene cages with biologically active groups has often been proposed (Mikawa *et al.,* 2001, Yu *et al.,* 2010, Bolskar *et al.,* 2003 , Sitharaman *et al.,* 2007 Fatouros *et al.,* 2006, Zhang *et al.,* 2010 , Shu *et al.,* 2009 , Shu *et al.,* 2008 and Fillmore *et al.,* 2011) . For example, the organic phosphonate modified Gd@$C_{82}O_2(OH)_{16}(C(PO_3Et_2)_2)_{10}$ has exhibited high affinity to bone (Yu *et al.,* 2010).

b.Radionuclides : Like Gadolinium other radionuclides can be encapsulated in fullerenes for either diagnostic or therapeutic purposes. The holmium-fullerenol, 166Ho*x*@C82$(OH)_y$ (*x* = 1, 2) , showed a blood pool residence period, higher than 1 h, contrary to classic metal



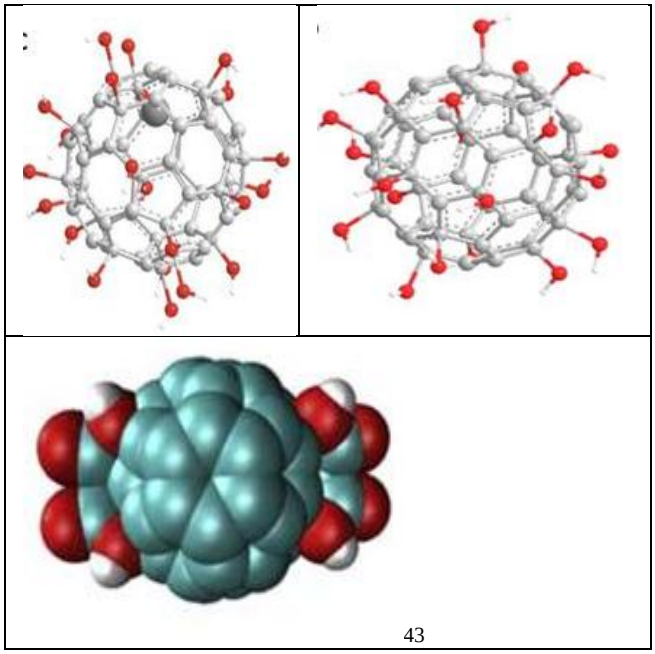


Figure 18 Depiction of (**39**) Gd@$C_{60}[C(COOH)_2]_{10}$ (Sitharaman *et al.,* 2004), (**40**) Gd@$C_{60}(OH)_x$ (Sitharaman *et al.,* 2004), (**41**) Gd@$C_{82}(OH)_{22}$ (Liu *et al.,* 2009 184), (**42**) $C_{60}(OH)_x$ (Liu *et al.,* 2009a), (**43**) $C_{60}[C(COOH)_2]_2$ (Li *et al.,* 2011).

chelates such as $Na_2[166Ho(DTPA)(H_2O)]$ and can reside up to 4 days in some organs such as liver and bones, showing a slow but steady clearance. This property allows to consider this endohedral as a possible new non-toxic radiotracer, suitable for prolonged time studies (Cagle *et al.,* 1999). 133Xe, another β-emitter, was entrapped into fullerenes through ion implantation technique (Watanabe *et al.,* 2003). The radiolabeled endohedrals were converted in their corresponding fullerenols 133Xe@C60$(OH)_x$ and 133Xe@C70$(OH)_x$, for cancer radiotherapy. However, a gradual release of xenon (10% after 5 days at 20°C) was observe due to polyhydroxylation. The polyhydroxylation process weakens the cage, decreasing the number of double bonds, and permits the gas release (Watanabe et al., 2005). In radioimmunotherapy, 212-Pb is used as β-emitting ,parent of the α-emitter 212-Bi. The use of Pb-chelates such as 212-Pb-DOTA leads to an accumulation of lead in bone marrow. The water-soluble 212-Pb@C60$[C(COOH)_2]_x$ avoided the accumulation but in this case 212-Bi (formed by β-decay) seems to be released from fullerene and causes damage by the β-decay (Watanabe *et al.,* 2005). Also 177Lu*x*Lu3−*x*N@C80 was synthesized as a new candidate in cancer radioimmunotherapy and it was demonstrated that, in this case, the fullerene cage is resistant to the 177-Lu β-decay, and so the toxic metal is not released. Then the corresponding fullerenol was conjugated to Interleukin-13 to specifically target glioblastoma multiforme tumor. Furthermore 177-Lu emits γ-rays too, and this allows to track the biological behavior of this endohedral by means of imaging (Shultz *et al.,* 2010). 99mTc is another useful radionuclide in nuclear medicine, but its half-life (6 h) is too short to permit the synthesis and the biological study of endohedral Tc@C2n derivatives. But some exohedral technetium fullerene conjugates have been prepared and 99mTc-labeled-C60$(OH)_x$ biodistribution and protein binding have been investigated following the radioactivity in dogs(Djordjevic *et al.,* 2011). Metallofullerene Liposome have been used for Contrast-Enhanced Molecular MR Imaging (Dellinger *et al.,* 2003, McFarland *et al.,* 2008, Podrez *et al.,* 2002, Amirbekian *et al.,* 2007, Nunn *et al.,* 1997). Atherosclerosis-targeting contrast agent (ATCA) incorporates a recently discovered metallofullerene MRIT1 contrast agent (Hydrochalarone®) and the specially oxidized lipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (oxPAPC) targeting inflammatory macrophage CD36 receptors in a self-assembled liposome construct (McFarland *et al.,* 2008).

Photoacoustic imaging : Recently, an attractive new therapeutic concept called 'acoustic-explosion' was suggested for some functionalized fullerenes, polyhydroxy fullerenes (PHF; $C_{60}(OH)_xO_yNa_z$) and carboxy fullerenes (CF; $C_{60}(C(COOH)_2)_3$). Concomitant with the exposure to low-intensity (< 10^2 W cm^{-2}) continuous-wave laser irradiation in the presence or absence of oxygen, sustained glowing of functionalized fullerenes was observed. When this process was performed in cancer cells, a faint pop was heard following irradiation, indicating the photoacoustic properties of functionalized fullerenes. To investigate their potential for cancer imaging, researchers then used a mechanical scanning photoacoustic system with a single transducer to collect the acoustic signal, and embedded it in a phantom together with functionalized fullerenes. After laser irradiation in a water tank, high contrast photoacoustic images of functionalized fullerenes were obtained. Similarly, the photoacoustic imaging experiment yielded promising results in tumor-bearing nude mice. Excellent contrast between tumor and non-tumor regions was shown after intratumoral injection of PHF, and the laser energy used was only one-third of the maximum permissible exposure level of 29.5 mJ cm^{-2} for a 785 nm pulsed laser (Krishna *et al.,* 2010 and Krishna *et al.,* 2010b). This new finding suggests potential applications of functionalized fullerenes for obtaining photoacoustic images .

Photothermal treatment : Recently, certain functionalized fullerenes, especially polyhydroxy fullerenes (PHF) and carboxy fullerenes (CF) were found to be heated to their ignition temperature by exposure to low-intensity (< 10^2 W cm^{-2}) continuous-wave laser irradiation, which was likely because of distortion of the symmetrical cage structure (Chen ***et al.,*** 2012). This heating property would be extremely advantageous for cancer therapy, when a negatively charged PHF coating on silica nanoparticles functionalized with positively charged amine groups was dosed to A549 cells and localized destruction of cells was induced by irradiation with a near-infrared laser (Krishna ***et al.,*** 2010b). .Moreover, when tumors were exposed to near-infrared light after injected with PHF nanoparticles, they decreased in cross-sectional area by an average of 32% within 2 hours of treatment, with only a blister visible 20 hours post-treatment. In addition, a concomitant faint audible sound was heard accompanied by bubble formation and bursting of the irradiated cells after exposure to a 785 nm laser. Thus, the rapid tumor shrinkage may be explained by both photothermal ablation and "acoustic-explosion" mechanisms (Krishna ***et al.,*** 2010).

Therapeutic Nuclear Medicine : While the potential of metallofullerenes on MRI was eveloped vigorously, research on another application of functionalized fullerenes to therapeutic nuclear medicine was also performed. Based on a similar principle, metallic radionuclides encapsulated in fullerenes can be protected in the empty carbon cage stably, eliminating any undesired toxicity induced by the leakage and catabolism of administered radionuclides.The availability of radio-fullerenes in therapeutic nuclear medicine was then proven by distribution and stability evaluations. Diener *et al.* prepared the α-emitting $^{212}Pb@C_{60}$ malonic acids for the first time and investigated the bio-distribution of the untargeted water-soluble radio-fullerene in mice and its stability during β^--decay of ^{212}Pb to ^{212}Bi. It was shown that $^{212}Pb@C_{60}$ malonate prevented the ^{212}Pb from accumulation in bone, attenuated the myelotoxicity of ^{212}Pb, and exhibited a rather slow clearance. This radio-fullerene also appeared stable in solution (about 36% of the ^{212}Bi released) (Diener *et al.,* 2007). The above results suggested that water-soluble fullerenes might be suitable as cancer radiopharmaceuticals.Effective targeted delivery of radionuclides is the primary challenge in radio-immunotherapy (RIT). By conjugating the radio-fullerenes with an antibody that is specific for receptors expressed preferentially on the cancer cells, radioisotopes can be delivered to cancer cells. After encapsulation of the β-emitter ^{177}Lu in a fullerene cage, an IL-13 peptide that targets an over-expressed receptor in glioblastoma multiforme tumors was successfully conjugated to the $^{177}Lu_xLu_{(3-x)}N@C_{80}$ (Michael et al., 2011). However, the efficiency of these radio-fullerenes for tumor targeting require further investigation in the living body or tumor cells.

Chemotherapeutics : Besides surgery, chemotherapeutics is another principal method for tumor therapy. However, the high toxicity and easily developed drug resistance considerably confine the chemotherapeutic effect. In recent years, functionalized fullerenes have been found to have some good characteristics to aid with antitumor treatments, and some of them have exhibited the potential for tumor inhibition (Liu *et al.,* 2009 ,Liu *et al.,* 2009 and Li *et al.,* 2011). In 2005, we found that gadolinium endohedral metallofullerenol ($Gd@C_{82}(OH)_n$ nanoparticles) can efficiently inhibit the growth of murine H22 hepatoma without obviously adverse effects on important organs, having nearly no direct toxic effect to tumor cells (Chen *et al.,* 2005), which is an advantage over conventional anti-tumor drugs. Zhu *et al.* (Zhu *et al.,* 2008) also tested the tumor-inhibitory effect of $C_{60}(OH)_x$ on the same model. In the $C_{60}(OH)_x$-treated group, significant tumor inhibition rates and reduced damage to liver were shown, in accordance to the histological results of inhibited tumor infiltration. Additionally, the anti-metastatic activities of fullerenol $C_{60}(OH)_{20}$ was observed in cancer metastasis models (Jiao *et al.,* 2010). Moreover, some fullerenes could increase the chemo-sensitization of tumor cells to chemotherapeutic agents and result in antitumor drug-mediated cell death, especially in some drug-resistant cancer cells (Jiao *et al.,* 2010 , Liang *et al.,* 2010 and Zhang *et al.,* 2009). Fullerene Liposome for Chemotherapy and Cancer Theranostics has also been used (Zakharian *et al.,* 2005 and Dellinge *et al.,* 2013). Anti-angiogenesis : Angiogenesis is one of the most important factors for the progression of tumor, by supplying tumors with oxygen and nutrients (Folkman *et al.,* 1971). As tumors can generate their own blood vessels from the surrounding vasculature (Wang *et al.,* 2010 and Ricc-Vitiani *et al.,* 2010), anti-angiogenesis treatments can inhibit growth and metastasis of various solid tumors (Kou et al., 2004).Meng *et al.* investigated the anti-angiogenic activity of $Gd@C_{82}(OH)_{22}$ and found that the nanoparticle can simultaneously down-regulate more than 10 angiogenic factors, which was confirmed *in vivo* by decreasing tumor microvessel density and lowering the nutrient supply to tumor tissues. A similar result was also obtained in another anti-angiogenesis study of $C_{60}(OH)_{20}$ (Jiao *et al.,* 2010 , Meng *et al.,* 2010 and Yin *et al.,* 2009). Fullerene compounds have been studied to hair re growth and hair loss due to alopecia (Zhou *et al.,* 2009). Recently C_{60} Fullerene has been tried as Synergistic Agent in Tumor-Inhibitory Doxorubicin Treatment (Prylutska *et al.,* 2014)

Modulation of oxidative stress : Reactive oxygen species (ROS) such as superoxide radical anion, hydrogen peroxide, singlet oxygen, and hydroxyl radical, which can cause oxidative stress, are considered to be an important part in the mechanism of carcinogenesis. ROS can induce lipid peroxidation, protein fragmentation, DNA damage, damage to tumor-suppressor genes and enhance the expression of proto-oncogenes. It has been reported that various carcinoma cells synthesize ROS at an elevated rate *in vitro*, and many tumors are under persistent oxidative stress *in vivo* (Jiao *et al.,* 2010). As water-soluble fullerenes showed high inhibitory activity on many kinds of tumor growth in mice, and it is well established that fullerenes and their derivatives possess a unique capacity for scavenging ROS (Yin *et al.,* 2009), the relationship between the two functions of the nanoparticles was investigated.

The ROS scavenging capability of $[Gd@C_{82}(OH)_{22}]_n$ was verified by electron spin resonance (ESR) (Yin et al., 2008), as well as in tumor tissues of $C_{60}(OH)_{20}$ treated mice (Jiao *et al.,* 2010), which meant that the antitumor mechanism of fullerenes may be related to its capacity to modulate oxidative stress in tumor-bearing models. On the other hand, Injac et al. found that fullerenol $C_{60}(OH)_{24}$ had protective effects on the primary organs (heart, liver, lung and kidney) against chronic toxicity induced by doxorubicin, and thus may be useful as a potential organo-protector for anticancer therapy (Injac *et al.,* 2008 , Injac *et al.,* 2009 Injac *et al.,* 2008a and Injac *et al.,* 2009a). These results may increase the feasibility of applying fullerenes to cancer chemotherapeutics.

Immunological activity : The water-soluble $C_{60}(OH)_{20}$ and $Gd@C_{82}(OH)_{22}$ nanoparticles have specific immunomodulatory effects on T cells and macrophages, and up-regulated the immune response *in vivo*, including the polarization of the cytokine balance towards Th1 (T-helper cell type 1) cytokines, which particularly increase TNF-α production, a very important factor helping to scavenge and kill tumor cells (Liu *et al.,* 2009 and Liu *et al.,* 2009a). These results indicated effective immunological mechanisms of functionalized fullerenes.

Possible immune-associated pathways by which $Gd@C_{82}(OH)_{22}$ nanoparticles inhibit the growth of tumors. in comparison to the untreated saline group, IFN-γ and TNF-α expression levels in mouse

tumor tissues increased markedly in the group treated with 0.5 mmol/kg/day $Gd@C_{82}(OH)_{22}$ nanoparticles. The $Gd@C_{82}(OH)_{22}$ nanoparticles injected in the abdominal cavity are mostly engulfed by macrophages and other phagocytes through phagocytosis, whilst a few enter the blood directly through the peritoneum or mesentery. The $Gd@C_{82}(OH)_{22}$ nanoparticles stimulate macrophages and T cells to release several kinds of cytokines, such as, IL-2, IL-4, IL-5, TNF-α and IFN-γ, which then triggers a series of signal pathways of immune responses and possibly promotes tumor cell apoptosis (Liu *et al.*,2009).

Zhang et al. found that $Gd@C_{82}(OH)_{22}$ could effectively activate dendritic cells (DCs) and macrophages, which are the most important antigen presenting cells (APC), as well as up-regulate the expression of co-stimulatory molecules and MHC molecules, thus enhancing the APC function of DCs and macrophages as illustrated by their capacity to activate allogeneic T cells. More evidence was also given to demonstrate the immune activity of $Gd@C_{82}(OH)_{22}$, which could increase the production of Th1 cytokines and promote macrophages secreting pro-inflammatory cytokines such as IL-6 and TNF-α (Yang *et al.,* 2010 and Wang *et al.,* 2011).

Moreover, there are few reports about the antigenicity of C_{60} derivatives. Chen *et al.* found that mice immunized with the fullerene-bovine thyroglobulin conjugates generated a population of fullerene-specific antibodies of the IgG isotype, including a subpopulation that was cross-reacted with a C_{70} fullerene (Chen *et al.,* 1998). The specificity of the monoclonal antibody for the C_{60} fullerene was determined by competitive inhibition, and the anti-C_{60} antibodies were able to interact with single walled carbon nanotubes, which was imaged using atomic force microscopy (Erlanger *et al.,* 2001 and Braden *et al.,* 2000).

Overcoming tumor resistance to chemotherapeutic drugs : For cisplatin-resistant cancer (CP-r) cells, $Gd@C_{82}(OH)_{22}$ alone had no obvious inhibition on tumor growth, although it has been indicated that the metallofullerene could improve the sensitivity of CP-r tumors to cisplatin, as an effective inhibition of CP-r tumors growth was observed following cisplatin plus nanoparticle treatment *in vitro* and *in vivo*. Transferrin-labeled experiments have shown that less intracellular transferrin is distributed in CP-r cells than in CP-s cells, indicating a possible defective endocytosis of the CP-r cells. Since nanoparticles have been used as drugs or gene carriers for cancer treatment, it was found out that $[Gd@C_{82}(OH)_{22}]_n$ nanoparticles reverse tumor resistance by enhancing the endocytosis of cisplatin via nanoparticle-mediated penetration through the plasma membrane of the CP-r cells, and this hypothesis was confirmed by increased cisplatin adducts in CP-treated CP-r cells. Restoring defective endocytosis and other related possible mechanisms for overcoming tumor resistance to cisplatin by using fullerenes could lead to new chemotherapies for cancer (Zhang *et al.*,2009). The fullerene derivative have also been used in autophagy-mediated chemo-sensitization (Shintani *et al.,* 2004 and Wei *et al.,* 2010).

Fullerenes for Medical Therapeutics : The anti-inflammatory properties of Luna's new fullerene compounds provide a host of opportunities for advancing medical treatments. Fullerenes are exceptional free radical scavengers, or antioxidants. They can intercept free radicals and neutralize them before they cause cellular harm for many diseases such as :

(i)Allergy : Mast cells (MC) are found in most tissues throughout the body and have traditionally been associated with initiating and propagating the allergic response. Fullerene derivatives are being investigated to inhibit diseases that are critically affected by MC responses. In simplistic terms, the noxious mediators released from MC when a person encounters something he/she is allergic to causes the familiar symptoms associated with allergic reactions. These symptoms range from annoying "hay fever" to life–threatening anaphylactic reactions leading to death. Indeed, most allergy medications are aimed at neutralizing (anti-histamines, H1-receptor blockers) or preventing MC responses. Fullerene derivatives may be capable of blocking this process and, unlike current medications, prevent the allergy reaction before it has a chance to proceed (Ryan *et al.,* 2007).

(ii)Asthma : There is substantial evidence that certain forms of asthma are critically dependent on MC and asthmatic attacks are often triggered by common allergens such as cat dander, pollen, etc. The underlying cause of these asthmatic attacks is due to MC mediator release. Under investigation are fullerenes to prevent MC activation in the lung so an asthma attack does not occur.

(iii)Arthritis : Mast cells play a critical role in the onset of arthritis.(Nigrovic *et al.,* 2005) Thus, as part of our overall strategy for designing fullerenes that interrupt MC-associated diseases, focus is also on investigating the effects they have on preventing arthritis.

Osteoporosis : Materials that set into solid, calcium-containing mineral products are of particular interest as such products can closely resemble the mineral phase of natural bone and are potentially re-modelable. The bone replacement materials are used for repairing fractured bone, strengthening cancerous bone, reinforcing osteoporotic bone, accelerated dental implant anchorage, and the like. There is a need for a bone replacement material having improved biocompatibility with natural bone. Further needs include a bone replacement material that facilitates the regeneration and growth of bone. Additional needs include a bone replacement material that is biodegradable.

In the treatment of osteoporosis and other bone disorders, it is desirable to deliver therapeutic compounds to the bone. However, it is known that certain substances, although therapeutic, are too toxic to be transported in the human body in free form. For example, fluoride anion (F–) is known to be an active therapeutic agent for osteoporosis, and the only known agent that can generate new bone matrix and new mineral from previously inactive areas. It both improves bone strength and helps prevent fractures. Yet it is too toxic to be administered in free form, such as by injecting NaF in aqueous solution intravenously.

One recently approved treatment for bone disease is a class of chemicals known as biphosphonates. Bisphosphonates bind to bone, slowing osetoporosis and allowing new bone to be formed. However, because this effect is temporary, bone mass is not substantially increased in the long term. Because new bone is not formed, bones are left weakened and prone to later injury. Hence, bisphosphonates alone are not entirely satisfactory. There remains a need for a suitable compound that inhibits bone resorption and promotes new bone formation so as to produce a net bone gain without adversely affecting the patient. Further, such a compound would be targeted to bone, permitting the release of a therapeutic agent at the site of the bone, and hindering any, potentially harmful release of the agent to the rest of the body.

A preferred method of synthesis of a fullerene-based bisphosphonated drug includes attachment of a water-solubilizing group, and attachment of a therapeutic agent. A plurality of fluorine atoms, as a therapeutic agent, are bound to one hemisphere the fullerene, thus hindering toxicity. Further it is believed that fluorine anion is released over time at the surface of the bone, due partly to the basic and nucleophilic environment of bone's surface .

It is known that fluorine anion generates new bone matrix and new mineral from previously inactive areas. It is envisioned that polyfluorobisphosphonated fullerenes, as bimodal drugs, can deliver the two bisphosphonate and F– components to bone in a single, non-toxic "package" that is easily absorbed in the gastrointestinal tract. Polyfluoro biphosphonated fullerene derivatives are being developed as bimodal drug for osteoporosis therapy (Gonzalez *et al.,* 2002) (because of preferential localization of fullerene derivatives in bones. However, the use of fullerenes as biological or pharmacological agents required immunological data and fullerene specific antibodies.

The fullerene specific anti-bodied has been developed by immunization of mice with a water-soluble C_{60} fullerene derivative conjugated to bovine thymoglobulin. The yield of fullerene-specific antibodies of the IgG isotype, showing that the immune system was diverse enough to recognize and process fullerenes as protein conjugates. A monoclonal fullerene-specific antibody has been isolated and characterized (Chen *et al.,* 1998). The sequences of its light and heavy chains have been reported (Braden *et al.,* 2007).

The effects of C60 on the receptor activator NFκB {RANK-} induced osteoclastogenesis and osteoclastic bone resorption were examined *in vitro* (Yudoh *et al*., 2009). Adjuvant-induced arthritic rats were used as an animal model of arthritis. Rats were divided into two subgroups: control and treatment with C60 at 1.0 μM. The left ankle joint was injected intra-articularly with water-soluble C60 (20 μl) in the C60-treated group, while, as a control, the left ankle joint in the control rats received phosphate-buffered saline (20 μl) once weekly for eight weeks. Ankle joint tissues were prepared for histologic analysis. C60 significantly inhibited the responses of osteoclast precursor cells to RANK ligand, including osteoclast differentiation and osteoclastic bone resorption *in vitro*. In adjuvant-induced arthritic rats, intra-articular treatment with C60 *in vivo* reduced the number of osteoclasts and alleviated bone resorption and destruction in the joints, while control ankle joints showed progression of joint destruction with time. These findings indicate that C60 downregulates the RANK-induced osteoclast differentiation and is a potential therapeutic agent for inhibition of osteoclastic bone destruction in arthritis (Yudoh *et.al*., 2009).

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