Medical Applications of Fullerenes and Metallofullerenes

by Lon J. Wilson

Since the discovery of fullerenes and the subsequent incorporation of metal atoms within their carbon cage in 1985, intense interest has focused on these unique molecules.[1] Research has greatly extended our knowledge of the chemical and biological properties of empty fullerenes, but comparatively little is yet known about endohedral metallofullerenes because of their low production yields (10^2-10^3 times lower than for empty fullerenes), low solution solubilities, difficult purification, and air sensitivity. However, recent advances in purification techniques and chemical derivatization of fullerene materials have made applications feasible for even endohedral metallofullerenes if very small amounts of material are required and if higher costs might be tolerated. Medicine represents a prominent field which satisfies both of these requirements.

Today, the most abundant empty fullerene, C_{60}, can be purchased for about $30/gram, whereas a pure sample of an endohedral metallofullerene such as [Gd@C_{82}] (@ denotes an internally-trapped gadolinium atom) costs ca. $1000/milligram to produce for research purposes. At this cost, pure samples of metallofullerenes are still novelty materials. Figure 1 compares the structures of empty C_{60} and Gd@C_{82} where the gadolinium atom is located off center.

Production and Chemical Reactivity

Bulk quantities of empty fullerenes were first produced by resistive heating of graphite in a helium atmosphere. The fullerenes in the carbon soot can be extracted into organic solvents, and the individual sizes (C_{60}, C_{70}, C_{84}, etc.) can be separated from one another by conventional chromatographic techniques. Impregnating the graphite rods with a metal salt (oxide, carbide or nitrate) and annealing at ca. 1000°C before burning the rods produces a small amount of metallofullerenes in the soot. Extraction of the soot with solvents such as CS_2 or o-dichlorobenzene, followed by HPLC (high pressure liquid chromatography) using specialized columns, is then used to obtain pure samples of endohedral metallofullerenes. M@C_{82} is usually the predominant species extracted, although multiple metal-atom species such as Y, Ho, or Er dimers, etc., and Sc trimer can also be formed and extracted in some cases. In general, a broad range of endohedral species containing from 60 to 200 carbon atoms and one or more metal atoms are formed, but most are insoluble.[2]

Typically, the encapsulated metal atom is an alkali metal, alkaline earth metal, Sc, Y, U, or a lanthanide metal, with the most unusual of these species being Sc_3N@C_{80} which has a nitride-bridged Sc_3N cluster inside a fullerene.[3] Most of the other metals in the periodic table do not form endohedral metallofullerenes, but rather form insoluble metal carbides and other unextractable materials. Fortunately, the lanthanide elements play a dominant role in diagnostic and therapeutic medicine, and hence lanthanometallofullerenes are attractive species for applications in medicine.

The synthetic organic chemistry of empty C_{60} and its derivatives is becoming an established field. Fullerenes undergo hydrogenation, halogenation, epoxidation, the formation of exohedral transition metal complexes, cycloadditions, carbene additions, alkylation and arylation. Fullerene derivatives of sugars, amino acids, polypeptides, estrogen, cholesterol and large proteins (BSA) are also known. Even antibodies to fullerenes have been produced recently.[4] While derivative chemistry of metallofullerenes has been demonstrated in a few cases, a general lack of material has hampered development of the field. These chemistries all attest to the stability of fullerenes with respect to cage opening and the ease of derivatization that make them and their metallofullerene counterparts so attractive as new biomedical materials.

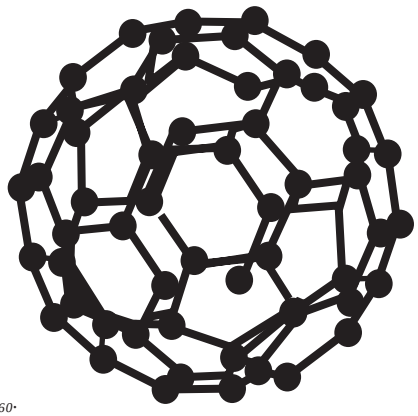


FIG. 1a. Empty C_{60}.

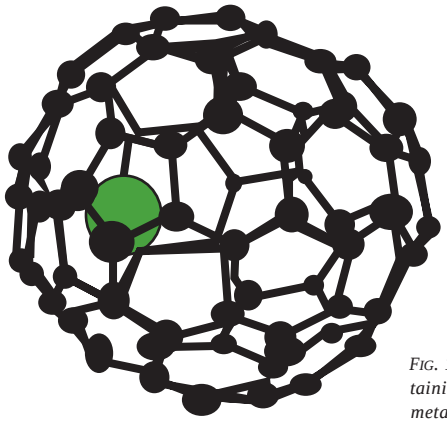


FIG. 1b. C_{82} fullerene containing an encapsulated metal atom.

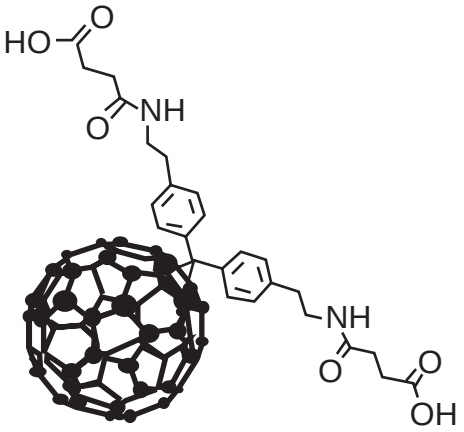


FIG. 2. A fullerene molecule that inhibits HIV protease.

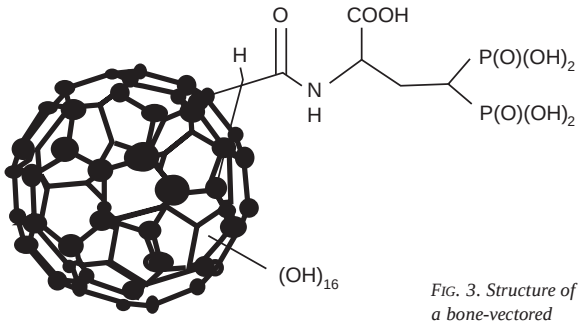


FIG. 3. Structure of a bone-vectored fullerene molecule.

Empty Fullerenes in Medicine

The first hint that fullerenes might have a future in medicine appeared in 1993 with the report that the bis(monosuccinimide) derivative of p,p'-bis(2-aminoethyl)-diphenyl-C_{60} shown in Fig. 2, is an HIV protease inhibitor.[5] Today the projected use of fullerene compounds in medicine is rapidly expanding.

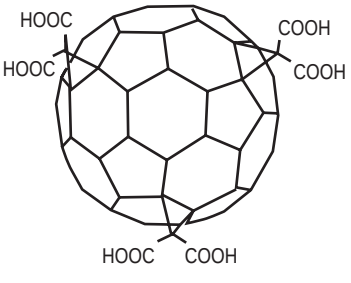
The first problem to overcome for medical applications stems from the extreme hydrophobicity of fullerenes. Underivatized fullerenes are soluble in aromatic solvents, but insoluble in water. To be medically useful, C_{60} and metallofullerenes must be derivatized with hydrophilic substituents. To date, the substituents of choice to produce water solubility have been either hydroxyl or carboxylic acid groups. In general, 12-16 hydroxyl groups or six carboxyl groups are needed to adequately solubilize C_{60} and [M@fullerene] molecules.

In addition to water solubilizing fullerenes, derivative chemistry can be used to produce fullerene-based drugs which target specific tissue. This is not easily accomplished, and only one success has been reported. Figure 3 displays a fullerene molecule derivatized with 16 hydroxyl groups and an appended arm with a terminal bisphosphinate moiety. The hydroxyl groups aid with water solubilization, while the bisphosphinate group is known to possess high affinity for hydroxyapatite (HAP) or bone tissue. *In vitro* HAP crystal growth inhibition studies for this bisphosphinate fullerene do, indeed, demonstrate high affinity and specificity for bone tissue; *in vivo* studies have yet to be performed.[6] More sophisticated tissue-targeting experiments, employing antibodies as the tissue vector, are on the near horizon.

Recent results indicate that fullerenes may have potential as biological antioxidants. Because of the large number of conjugated double bonds that are readily attacked by radical species, C_{60} has been referred to as a radical sponge, and facile addition of up to 34 methyl radicals to C_{60} has been reported. In fact, C_{60} is probably the world's most efficient radical scavenger. Because of this property, C_{60}, derivatized by hydroxylation or carboxylation, provides antioxidant and neuroprotective ability. Perhaps the most promising medical use of fullerenes to date is the finding that fullerene antioxidants can extend the lives of transgenic mice that carry a defective copy of the gene encoding for human superoxide dismutase (SOD1) found in familial cases of amyotrophic lateral sclerosis (ALS), commonly known as Lou Gehrig's disease. Mice treated with the carboxylfullerene derivative shown in Fig. 4 have a delay in symptoms of 10 days and live approximately 8 days longer than untreated mice.[7]

In yet another potential medical application, a C_{60}-PEG (polyethylene glycol) derivative has been proposed as a photodynamic therapy agent. The use of fullerenes as photosensitizers has been often advocated for this purpose, due to their ability to convert triplet oxygen ($^3O_2$) to highly-reactive and tissue-killing singlet oxygen ($^1O_2$). Using the C_{60}-PEG complex, photodynamic therapy has been successfully performed on mice with Meth A fibrosarcoma tumors.

Research into the biological effects of fullerenes has only just begun. However, preliminary studies have indicated that fullerenes and their derivatives are relatively non-toxic *in vivo*. For example, polyhydroxylated C_{60} (a fullerol) demonstrates an LD-50 in rats of 1.2g/kg, with no effect observed from intraperitoneal administration of <100mg/kg. It should be recognized, however, that the biodistribution, pharmacokinetics, and toxicity of fullerenes and metallofullerenes will depend on the derivative used to make them water soluble. To date, no toxicity information is available for a metallofullerene, although there is little reason to expect different toxicities than for empty fullerenes.



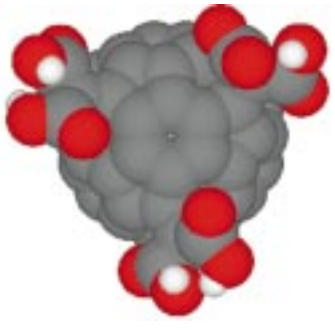


FIG. 4. Structure of the C_{60}-based carboxylfullerene antioxidant and ALS drug. This chiral structure, as viewed down the C_3 symmetry axis, contains 6 carboxylic acid groups (from 3 malonic acids) attached to the C_{60} sphere to make it water soluble.

Metallofullerenes in Medicine

The purification of endohedral metallofullrenes in reasonable quantities (milligrams) is difficult, mainly because the metallofullerene content of the extract is limited (ca. 1%) and because their solubility in conventional solvents is even lower than for various higher empty fullerenes. It took nearly six years for [La@C_{82}] to be

isolated and purified in quantity after it was first discovered.

Electron paramagnetic resonance (EPR) spectroscopy is one of the most powerful methods for establishing the electronic structure of endohedral metallofullerenes, since most of the mono- and tri-metallofullerenes studied have exhibited hyperfine splitting from electron-spin nuclear-spin coupling. Epr spectroscopy indicates a formal [M^{2+}-(fullerene cage)$^{2-}$] or [M^{3+}-(fullerene cage)$^{3-}$] electronic structure for lanthanide monometallofullerenes. Thus, overall, metallofullerenes are neutrally-charged molecules. STM images and a synchrotron X-ray power diffraction study of [Y@C_{82}] have demonstrated that the Y^{3+} ion inside a charged C_{82}^{3-} cage is strongly bound to one side of the cage, producing a molecule with a permanent dipole moment.

Although available only in small quantities now, endohedral metallofullerenes are unique molecules with unusual properties which may render them especially useful in medicine. Foremost among these properties are: (1) an all-carbon shell with a large surface area (≥ 200 Å^2); (2) a hollow core (ca. 7-8 Å diam) capable of accommodating up to three lanthanide ions; (3) a non-toxic and non-metabolizable inert molecular structure with K_{dissoc} = 0 for the internal metal ions; (4) an overall neutral charge; (5) a proven chemical reactivity of the surface carbon atoms for derivatization purposes; and finally, (6) a proven clearance of a metallofullerol species from a mouse. This set of properties distinguish metallofullerenes from all other methods for delivering metal atoms *in vivo* for medical purposes. To date, metallofullerenes have shown special promise as novel diagnostic and therapeutic radiopharmaceuticals, as well as new proton magnetic resonance imaging (MRI) contrast agents.

MRI Contrast Agents

Contrast enhancement agents have become a routine, if not indispensable, part of the MRI diagnostic process. These unique pharmaceuticals, usually composed of paramagnetic lanthanide or transition metal ion complexes, decrease the NMR relaxation times of nearby proton nuclei of H_2O molecules, leading to brighter images and enhanced contrast between areas containing the contrast agent and the surrounding tissues. The most popular of these are the gadolinium(III) chelate compounds of DTPA, DO3A, DOTA and other variations of these linear and macrocyclic ligands. Direct Gd^{3+}-OH_2 chemical bonds, which exchange rapidly with other bulk H_2O molecules, produce the mechanism whereby unpaired 4f electrons on Gd^{3+} relax the proton nuclei of many nearby H_2O molecules. Present MRI contrast agents perform well under most routine circumstances and experience with millions of doses of Magnevist (the N-methylglucamine salt of [Gd(DTPA)(OH_2)]$^{2-}$) has shown it to be very safe. However, better contrast agents are still needed for certain imaging applications.

Fullerenes that contain a paramagnetic lanthanide ion such as Gd^{3+} would have a number of important advantages as MRI contrast agents. The paramagnetic Gd^{3+} ion provides the unpaired electron density needed to decrease the relaxation time of nearby H_2O proton nuclei, while the fullerene cage protects the lanthanide from chemical attack and sequesters the toxicity of a naked Gd^{3+} ion. Once trapped inside the fullerene cage, the lanthanide ion is protected from the external environment, and the resulting lanthanofullerene is stable with respect to dissociation in even the most extreme chemical environments. Empty fullerenes are known to be stable with respect to cage opening reactions even in hot concentrated sulfuric and nitric acid.

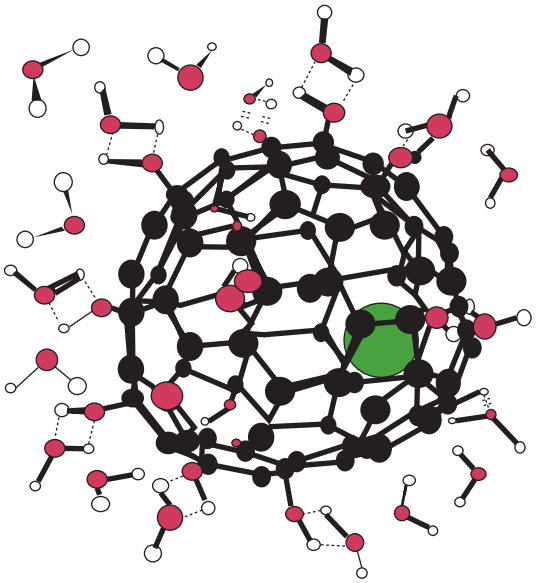


FIG. 5. A water proton relaxation process for [Gd@$C_{82}(OH)_x$] with a S = 1/2 fullerene cage. Affected water molecules are shown hydrogen bonded to cage hydroxyl groups.

In addition to their inherent stability, lanthanide-containing fullerenes behave chemically somewhat like empty fullerenes. Using techniques now being established for empty fullerenes, it should be possible to produce a wide range of lanthanofullerene-labeled derivatives for custom-designed contrast agents. For example, a water-soluble polyhydroxylated Gd^{3+}-metallofullerene, [Gd@$C_{82}(OH)_x$], has been synthesized by the tetrabutylammonium hydroxide (TBA(OH)) phase-transfer reaction of Scheme 1.

$$Gd@C_{82} + OH^-_{(ex)} \xrightarrow[TBA(OH)]{toluene/H_2O} [Gd@C_{82}(OH)_x] \quad (1)$$

The proton magnetic relaxivity (T_1) of [Gd@$C_{82}(OH)_x$], measured using nuclear magnetic resonance dispersion (NMRD), reveals that the Gd^{3+}-metallofullerol has a very high relaxivity of R_1 = 20 m^{-1} s^{-1} at 40°C and 20 MHz[8] which is five times the relaxivity of current commercial contrast agents such as Magnevist, ProHance, or Omniscan. This is an astounding result for a caged Gd^{3+} ion that appears to have no access to water for Gd^{3+}-OH_2 bond formation.

One working hypothesis is that this observed high relaxivity is due to the electronic structure of the paramagnetic (S = 1/2) metallofullerene cage in [Gd^{3+}@$C_{82}(OH)_x^{3-}$] and not directly to the f-orbitals of the encapsulated Gd^{3+} ion. Figure 5 suggests a process for [Gd@$C_{82}(OH)_x$] which could simultaneously relax the protons of many hydrogen-bonded H_2O molecules on the 200 Å^2 paramagnetic metallofullerene surface. This relaxation mechanism is unique when compared to that of conventional Gd^{3+} chelate compounds, suggesting that metallofullerenes could form the basis of an entirely new class of contrast agents.

Preliminary biodistribution studies indicate that the hydroxylated metallofullerene compounds have biodistributions that may make them useful as blood-pool imaging agents for MRI cardiography or angiography. It also seems likely that this biodistribution can be further modified or tailored to a specific application by simply altering the nature of the chemical substituent on the fullerene surface.

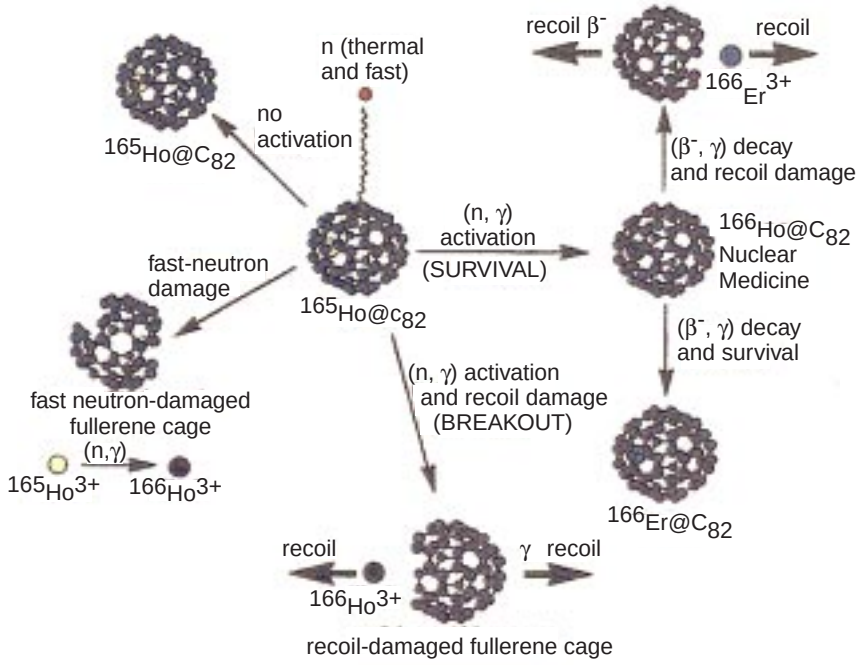


FIG. 6. Possible outcomes of irradiating a metallofullerene in a neutron flux. Not all outcomes are equally likely. (Reproduced with permission of Chem. Phys. Lett., **308**, *329 (1999).)*

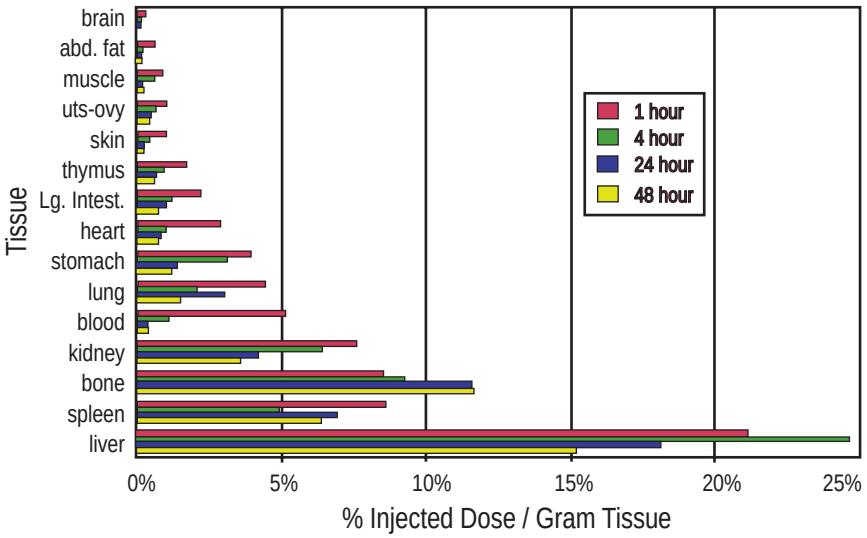


Fig. 7. Biodistribution of $^{166}Ho@C_{82}(OH)_y$ in BALB/c mice at 1, 4, 24, and 48 h after injection. (Reproduced with permission of Proc. Natl. Acad. Sci. USA, **96**, *5182 (1999).)*

Radiotracers and Radiopharmaceuticals

Another potential application for metallofullerenes is in the field of nuclear medicine. Current radiopharmaceuticals employ small quantities (nanograms or milligrams) of drugs containing specially-chelated radioisotopes of metals for imaging or therapeutic applications. The chelating ligands prevent direct binding of the toxic metal ions with serum components and tissue by providing a thermodynamically-stable molecular environment. A major concern with these drugs, however, is their *in vivo* kinetic instability, which can allow the release of small amounts of toxic radioactive metal ions. In comparison, metallofullerenes provide a unique alternative to chelating compounds because of their resistance to metabolism and their high kinetic stability. Thus, metallofullerenes may be useful as a new, more stable alternative for transporting radiometals *in vivo*.

The nuclear chemistry of radioactive holmium metallofullerenes has been examined in detail because (1) ^{165}Ho is naturally monoisotopic and (2) ^{165}Ho has a simple primary neutron activation/decay as shown by Scheme 2.

$$^{165}Ho \xrightarrow[\sigma = 61.2\ \text{barn}]{[n,\gamma]} {}^{166}Ho \xrightarrow[t_{1/2} = 26.8\ \text{h}]{\beta^-,\gamma} {}^{166}Er \quad (2)$$

In addition to its convenient nuclear properties, ^{166}Ho also has shown promise for therapeutic nuclear medicine (β– emission) and diagnostic imaging (γ emission). This established nuclear medicine use for holmium makes its metallofullerenes an appealing general model for metallofullerene-based radiopharmaceuticals.

There are several possible outcomes of irradiating holmium metallofullerenes in a neutron flux. These processes are outlined in Fig. 6. In the most likely event, the sample is unaffected by neutron bombardment, and metallofullerene integrity is preserved. For those metallofullerenes that do interact with neutrons, several subsequent events can occur.

The ideal metallofullerene neutron-irradiation pathway is one in which the heavy-metal center becomes activated and remains confined within the carbon cage. Subsequent decay of the metal radionuclide to a stable isotope (also remaining within the cage) completes the activation/decay cycle. This pathway is most desirable for nuclear medicine, since it results in high-yield activation of the metallofullerene materials and precludes *in vivo* metal release. Unfortunately, other contributing neutron-activation/decay processes can drastically reduce the yield of surviving metallofullerene, as also summarized in Fig. 6. Under certain conditions, however, 20-30% of a $[Ho@C_{82}]$ sample survives to become a precursor nuclear medicine.[9] This survival rate produces sufficient ^{166}Ho activity for *in vivo* radiotracer experiments. In the first such study, a sample of water-soluble $[^{165}Ho@C_{82}(OH)_x]$ was isolated, neutron activated to produce $[^{166}Ho@C_{82}(OH)_x]$, and injected into mice.[10] Monitoring the ^{166}Ho γ-decay, a complete biodistribution study of the metallofullerol material was conducted by dissection radioanalysis. Key findings, as summarized in Fig. 7, demonstrate that the radiotracer (1) lingered in the blood-pool for the first hour with nearly total clearance thereafter, (2) concentrated slowly over time in bone tissue without elimination,

and (3) localized in the liver with clearance of ca. 20% over the first five days.

This experiment demonstrates the feasibility of using water-solubilized metallofullerene radiotracers to monitor the fate of fullerene-based materials in animals and suggests that water-solubilized fullerene materials may be useful components in drug design. For example, a blood-pool MRI technology might be developed from a [Gd^{3+}-fullerol] and [$^{166}Ho^{3+}$-fullerols] could be ideal bone-seeking agents for bone-marrow ablation in the treatment of myeloma. Undoubtedly, many more potential medical applications will suggest themselves as we discover more about these fascinating new materials.

Conclusion

Empty fullerenes and endohedral metallofullerenes bring to medicine novel, 3-dimensional carbon structures that can be made water soluble, nontoxic, and tissue or mission selective. In particular, endohedral metallofullerenes provide a new class of compounds for delivery of metal ions such as lanthanides in activated form for nuclear medicine and non-activated form as contrast media. Forthcoming breakthroughs in the synthesis and manipulation of these compounds promise lower cost, more sophisticated analogs capable of challenging present market-place technologies. ■

Acknowledgement

The original research reported in this article was supported by the Robert A. Welch Foundation and the National Institutes of Health.

References

1. H. W. Kroto, J. R. Heath, S. C. O'Brien, R. F. Curl, and R. E. Smalley, *Nature*, **65,** 162 (1985).
2. M. D. Diener and J. M. Alford, *Nature*, **393**, 668 (1998).
3. S. Stevenson, G. Rice, T. Glass, K. Harich, F. Cromer, M. R. Jordan, J. Craft, E. Hadju, R. Bible, M. M. Olmstead, K. Maitra, A. J. Fisher, A. L. Balch, and H. C. Dorn, *Nature*, **401,** 55 (1999).
4. B.-X. Chen, S. R. Wilson, M. Das, D. J. Coughlin, and B. F. Erlanger, *Proc. Natl. Acad. Sci. USA*, **95,** 10809 (1998).
5. S. H. Friedman, D. L. DeCamp, R. P. Sijbesma, G. Srdanov, F. Wudl, and G. L. Kenyon, *J. Am. Chem. Soc.*, **115,** 6506 (1993).
6. K. A. Gonzalez, Ph.D. Dissertation, Rice University (1999).
7. L. L. Dugan, D. M. Turesky, C. Du, D. Lobner, M. Wheeler, C. R. Almli, C. K.-F. Shen, T.-Y. Luh, D. W. Choi, and T.-S. Lin, *Proc. Natl. Acad. Sci. USA*, **94,** 9434 (1997).
8. D. W. Cagle, Ph.D. Dissertation, Rice University (1998).
9. T. P. Thrash, D. W. Cagle, J. M. Alford, K. Wright, G. J. Ehrhardt, S. Mirzadeh, and L. J. Wilson, *Chem. Phys. Lett.*, **308,** 329 (1999).
10. D. W. Cagle, S. J. Kennel, S. Mirzadeh, J. M. Alford, and L. J. Wilson, *Proc. Natl. Acad. Sci. USA*, **96,** 5182 (1999).

About the Author

Lon J. Wilson *has been a professor of chemistry at Rice University for more than 20 years. Dr. Wilson has co-authored nearly a hundred papers in the fields of bioinorganic and biomedical materials chemistry. He and an industrial collaborator, Dr. Michael Alford of TDA, Inc., in Wheat Ridge, Colorado, have formed an industrial-academic partnership for the purpose of scientific discovery and the commercialization of fullerene-based materials in medicine.*