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Review Article

Pristine Nanocarbon-based Fullerene-like Material Toxicity and Biocompatibility (Part 2 in the series: Will Nanocarbon Onion-Like Fullerenes Play a Decisive Role in the Future of Molecular Medicine?)

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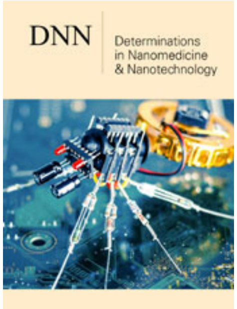

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Abstract

Outcomes of in vivo and in vitro biological studies of nanocarbon materials are varied and at times contradictory. Research suggests that nanocarbon materials (including pristine fullerenes) pose a risk to living systems. However, overall outcomes using pristine single- and multi-walled nanocarbon fullerenes have been favorable and suggest biocompatibility. Variability in research outcomes results from not only the interaction of the material with biological systems but also the quality and consistency of fullerene material (such as, inconsistencies in purity, structure, processing, and functionalization of fullerenes) that produce differences in characterization and properties even within the same batch. Due to the omnipresence of nanocarbon-based material in the environment, it should be considered that biological systems long ago developed ways to detoxify and or exploit nanocarbon-based material. The main focus of this paper is the factors contributing to relative biocompatibility and toxicity of fullerene material and the variations in their absorption, biocoronation, degradation, and elimination. A review of more current research suggests that theoretical or in vitro generalizations regarding any biological hazards of fullerene material are speculative and inaccurate; their biological interactions should be considered and assessed in vivo. If factors affecting the relative toxicity of fullerenes and consistency in their production are addressed, valuable research could move forward to explore and unleash the potential applications and benefits of fullerenes in living systems.

Keywords: Allotrope; Aromatic ring; C_{60}; Cyclic native aggregation; Dielectric property; Hormesis; Fullerene; Mitochondria; Mitochondrial QED; Nanocarbon; OxPhos; Pharmacophore; Pi clouds; Shungite; Quantum electrodynamic

Abbreviations: ADME: Absorption, Distribution, Metabolism and Excretion; CNO: Carbon Nano-onion; DMSO: Dimethyl Sulfoxide; EMF: Electromagnetic Field; EPO: Eosinophil Peroxidase; IVD: Intervertebral Disk; LPO: Lactoperoxidase; MPO: Myeloperoxidase; NOLF: Nanocarbon Onion-Like Fullerene; OXPHOS: Oxidative Phosphorylation; QED: Quantum Electrodynamics; ROS: Reactive Oxygen Species; THF: Tetrahydrofuran

Preface

The following preface is from the first in a series of articles by Bourassa & Kerna on nanocarbon onion-like fullerenes, titled: *Will Nanocarbon Onion Like Fullerenes (NOLFs) Play a Decisive Role in the Future of Molecular Medicine? Part 1. Foundation in Fullerenes: Theoretical Application of NOLFs in the Quantum Cell.* The preface is provided verbatim, herein, so the concepts in Part 2 may be readily grasped and the discussion proceed without having to refer to or review Part 1; however, a review of Part 1 is favored.

The biology underlying medicine is undergoing a subtle but radical paradigm shift. Investigations into the quantum cell have been gaining acceptance. At some point, there must be a paradigm shift in medicine to reconcile with the emerging realization that life's cellular machinery teeters on quantum criticality between order and chaos. Thus, an understanding of the detailed mechanisms behind this apparent self-organized criticality [1] becomes necessary in the prevention and treatment of disease.

Biology, based on classical physics, has fallen short in explaining how the highly organized molecular machinery (taking care of myriads of complex processes such as DNA replication, protein synthesis, cell division, and metabolism) can operate with perfect timing and precision in a healthy cell. The electrodynamics of the quantum cell provides the perpetual and precise transfer of charges throughout the system for perfect execution of biochemical tasks [2]. To more fully fathom the following exploration, it is helpful to be acquainted with concepts of the quantum cell, quantum electrodynamics, and electromagnetic fields in biologic systems as well as protein conformational change, structured cell water, and alternative cell physiology. [3]

Introduction

Reviewed in this research were published experimental and clinical studies on nanocarbon-based materials including fullerene-like materials with a spotlight on their production methods, absorption, distribution, degradation, and elimination in biological systems. Online databases referenced included PubMed, Medline, and Google Scholar to identify relevant articles regarding biocompatibility, toxicity, biological effects, and medical applications of C_{60}, fullerene, nanocarbon, nano onion-like fullerene, multi-walled nanocarbon fullerene, onion-like carbon, shungite, and graphene-based nanocarbon.

Pathways of fullerene exposure

The three most significant pathways to incidental and intentional fullerene material exposure and entry into the human system are inhalation, dermal exposure, and oral ingestion. There is a concern among researchers and users that nanocarbon-based material, such as fullerenes, are not biodegradable and remain a risk for biological systems. Detoxification and elimination pathways primarily involve the liver, intestinal tract, and renal system, but the mechanisms are unclear. If any detrimental effects effects are occurring in these pathways, it is reasonable that evidence of such can be seen. Due to their relative small size, nanomaterials are thought to easily bypass natural tissue barriers and penetrate cells and subcellular compartments where any adverse effects might be noted. However, size alone is not necessarily the determining factor in biodistribution or toxic potential. Surface modification and physiochemical properties have a significant effect on both.

Fullerenes and the living system (biocoronation, degradation, and elimination)

The interaction of engineered nanomaterials in biological settings results in the rapid coating of these molecules with proteins, lipids, and other molecules creating a biocorona [4]. This biocorona contains a hard corona comprised of a first layer of biomolecules that interact with the nanoparticles; then, acquires a second layer (that interacts with the first layer) termed the soft corona [5,6]. As these nanoparticles travel through various biological compartments, they enzymatically develop changes in physical parameters while retaining a memory of their journey [7]. Little is known about fullerene-biocorona tissue interaction.This lack of understanding regarding fullerene-biocorona tissue interaction remains a research challenge in explaining the biological effects of fullerenes and their modification for therapeutic applications. Furthermore, the biocorona likely plays an important role in modulating cellular uptake of nanocarbon materials [4]. The use of bovine albumen to create hydrophilic fullerene solutions for research is one such biocorona application [4,5].

Fullerenes and the respiratory system

Many of the various inhaled nanocarbon-based materials do not appear to result in lung pathology nor do they appear to remain in the lungs for extended periods. A detailed discussion of all the possible fates of nanocarbon material–specifically, nanocarbon onion like fullerenes (NOLFs)–is beyond the scope of this paper. Still, it should be mentioned at this point that most of the material is likely expelled through mucociliary transport and expectorated or swallowed (thus entering the digestive tract). Nanocarbon-based materials do not appear to adversely impact lung surfactant proteins or lipids. However, nanocarbon material may rapidly create a lung-based biocorona [4]. Any remaining NOLF material is likely phagocytized by immune cells in the lungs, such as alveolar macrophages which have been shown to phagocytize graphene-based materials [4]. Thus, long-term exposure to simple fullerenes and NOLFs may not result in granuloma or fibrotic changes in the lungs as some research into graphene-based nanocarbons has suggested. The lack of fibrosis and granuloma formation in the lungs is further supported by research regarding biocompatible nanocarbon materials [8].

Fullerenes and the immune system

The concern of biological persistence exhibited by other types of nanoparticles is absent in fullerenes. Nanocarbon based materials are biodegradable. The innate immune system is involved in their enzymatic digestion. The presence of a biocorona does not prevent degradation [4]. Several complementary pathways and cell types are engaged in degrading fullerenes. These degradation pathways include neutrophil myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO), and macrophage peroxynitrite [4]. The uptake of fullerene materials by these cells of the innate immune systems may also contribute to the biological benefits of fullerenes. Much of the research regarding such has been performed with functionalized rather than pristine fullerenes which are known to influence solubility and tissue interaction. Nevertheless, antioxidative properties and enhancement of cellular oxidative stress management may be responsible for the majority of detected anti-inflammatory effects [4,9,10].

Fullerenes and the GI system

Relatively little is known about the uptake of fullerene material in the GI tract. Many researchers and self-experimenters presume the absorption of pristine fullerenes is related to its hydrophobic nature in association with lipid micelles. The Baati study used olive oil to gavage the rats [11], but the reasoning was solubility, not absorption. Regardless, this has led to a perception by many self-experimenters that oral fullerene dosing should be in an oil base. However, while micelle pathway absorption is possible, it may not be the primary pathway. Nanoparticle absorption occurs without association with fat micelles. Research with water-soluble fullerenes supports non-micelle absorption mechanisms.

From the time fullerene material enters the mouth, it is subject to biocoronation and interaction with immune cells. If the surface of the nanoparticle, such as a pristine NOLF, has any shell defects, it may be subject to surface modification in the harsh pH environment of the stomach [8]. Once in the small intestine, it is subject to further biocoronation. Uptake is in association with the M cells of Peyer's patches, gut-associated lymphoid tissue, and epithelium (via limited transcellular or paracellular transport to the lamina propria) where NOLF has considerable interaction with immune cells of the gut's lymph system. Nanoparticle uptake is about 2-3% [8,12]. Fullerenes that are not absorbed are free to interact with the gut microbiome. This interaction results in changes to intestinal flora which may reduce inflammation and favorably influence multiple organ systems of the host [8,13].

Discussion

Since the earliest days of fullerene research into their application in living systems and continuing today, the biocompatibility versus toxicity of fullerenes remains a fundamental and paramount issue. The following discussion addresses the biocompatibility and toxic risks of fullerenes; also, the potential benefits of fullerenes are described and listed.

Biocompatibility and toxicity of fullerenes

Despite considerable experimental work to advance nanotechnology and its applications, the understanding of the occupational, health, and safety aspects of engineered nanomaterial remains in a developmental stage [14]. Not all nanocarbon and fullerenes are engineered. Fullerenes and other fullerene-like or fullerene-containing material occur frequently in nature [15]. Quite often, the evidence of toxicity demonstrated in vitro is not evidenced in vivo suggesting a more complex interaction in living subjects than demonstrated in the Petri dish. Nanocarbon-based materials, including fullerenes, have long been common in the environment. Thus, it follows that living systems, including humans, would have developed defense mechanisms (against any threat posed by such) [4] and or learned to exploit their physiochemical properties [3]. Some of these carbonaceous materials, such as shungite [16] and activated carbon [17], have found commercial and medicinal applications. Other carbonaceous materials are by-products of combustion and are generally considered environmental pollutants. Engineered single- and multi-shelled fullerenes have a somewhat different character and association due to their commercial use and medical potential.

The concerns of fullerene toxicity do not include biological persistence as their average elimination is under thirty days [18]. The frequency of fullerenes in nature (e.g., camp fires, candles, volcanic activity, and shungite to name just a few) suggests that living systems have developed protection against nanocarbon material toxicity. This proposed protection is supported by research demonstrating the uptake and degradation of nano materials via the innate immune system with little in vivo toxicity observed [4]. However, the absorption, distribution, metabolism, and excretion (ADME) profiles of various fullerenes requires further research. The intrinsic characteristics of fullerenes as well as their distribution and metabolism/degradation are thought to be altered by the absorption of proteins and other biomolecules in their biocoronation as they pass through organ systems [4].

Fullerene production methods

Several studies have suggested that pristine and functionalized fullerenes pose toxic risks to biological systems, and this possible toxicity presents challenges and obstacles to their use as pharmacophores [19]. Per contra, residues may have resulted in the attempts to obtain pure fullerenes for testing from fullerene soot or, at least, such residues may have contributed to toxicity in those studies. The use of tetrahydrofuran (THF) in preparing fullerene colloids is another source for certain toxic effects [20-22]. A 2013 paper by Orlova [23] provided a synopsis of fullerene toxicity studies as well as their association with medical applications.

Researchers are now more aware of the importance of consistent quality of fullerene material and attempt to reduce or eliminate contaminants. This reduction of contamination has resulted in a consensus among researchers and users that fullerenes, including NOLFs and their biologically functionalized derivatives, are well-tolerated in biological systems [24-30]. The inherent hydrophobic nature of pristine fullerenes has presented challenges to aqueous-based research in biological and biomedical applications [31]. This hydrophobicity of pristine fullerenes has resulted in the adoption of delivery methods (such as encapsulation and microencapsulation, lipid suspensions, use of organic cosolvents, and chemical functionalization by attaching various ligands) to increase hydrophilic solubility [24] which subsequently contributed to differing biocompatibility of fullerenes reported among researchers. Fullerenes are commonly produced in a "soot" form with other carbonaceous material. One of the first steps researchers perform in obtaining a "pure" fullerene sample is often accomplished by the Soxhlet method via the use of different solvents including toluene. Toluene is one such mitochondrial contaminant or toxic residue found in many nanocarbon materials used in biological toxicity studies [32,33], and continues to be used as evidenced in more recent papers [34]. Dimethyl sulfide (DMSO) employed in another study showed some toxicity to liver cells [35].

Nanocarbon-based fullerene-like material is varied with similar and differing toxicity and biocompatibility based on their different characteristics and properties. A few studies, primarily in vitro, have demonstrated toxicity of some fullerene and graphene-based nanocarbon materials. There are several hypothesized causes of toxicity; however, the most common contributors to toxicity in carbonaceous materials are contaminants, shell modification, shell charge, and structural shape. These contributors can be modified by hard and soft biocoronation with proteins, lipids, and other biomolecules.

Frequently, various toxic contaminants are a result of harsh and toxic organic solvents utilized to:

1. harvest the desired carbon structure
2. "functionalize" it for better absorbability or
3. produce specific hydrophilic carbon structures for certain applications.

Pristine, unmodified fullerenes (nonfunctionalized fullerenes) eliminate many of the toxicity inducing factors. However, some residual contaminants from the cleaning process may skew the physical characteristics and biological properties of fullerenes [31]. A major concern expressed by nanocarbon material researchers is there is little consistency in quality among batches of fullerenes even from the same supplier. Another issue is purity. Trace contaminants can be introduced in cleaning or producing a soluble product not only by manufacturers but also by researchers. Production methods produce relatively low yields of the desired carbon structure, and other preexisting contaminants must be removed during the production process. Carbon sheets or tubes with "sharp" edges can rupture delicate cell membranes; the spherical structure of fullerene-like material avoids this problem. Thus, end production biocompatibility and toxicity should be assessed for each manufacturing method or when functionalizing for specific applications.

Fullerenes and ROS production

It has been reported frequently that reactive oxygen species (ROS) production contributes to fullerene toxicity. Rarely mentioned is that all fullerenes behave like electron deficient alkenes with a strong affinity for electrons [36]. A molecule that expresses an affinity for electrons fits the definition of an oxidizer, not an antioxidant. At least one author has described this "fullerene paradox" [37]. At present, neither ROS production nor antioxidant properties present an adequate explanation for their various benefits without evidenceof pathology or adverse effects in their interaction with biologic systems.

This electron affinity seems to support findings of many researchers reporting ROS production by fullerenes especially in vitro testing but ignores ROS hormetic effects that benefit adaptation and survival. The inconsistent results among various researchers reporting on ROS production coupled with the lack of evidence of significant adverse effects of in vivo tests suggest that pure, pristine fullerene material-generated ROS production does not seem to pose a significant risk to biological systems. Cellular responses to UV excitation of C_{60} embedded in surfactant micelles produce ROS which suggests toxicity. However, aggregates of C_{60} do not produce ROS. UV photons excite C_{60} from its ground singlet state to a triplet state which is an essential intermediate in energy and electron transfer in the formation of ROS. (This ROS formation is utilized in cancer phototherapy for the destruction of surface tumors.) The collection of C_{60}-aggregated molecules quickly accelerates the excited triplet-state decay that results in blocking or preventing ROS formation [38]

The production of ROS is the most commonly reported finding suggesting fullerene toxicity. However, most of these studies are in vitro which expose cells and their organelles to supra-physiologic oxygen torr not found in cell systems *in vivo*. Thus, in whole organism systems, any interpretation of responses to biomarkers of oxidation and ROS effects in vitro are questionable [39]. While all biomarkers of oxidation, ROS, and associated damage indicate potential for toxicity, the preponderance of in vivo studies demonstrates little or no long-term toxic effects suggesting that there may be other explanations for the ROS findings or that the ROS production has a beneficial or adaptational advantage for certain cells [9,11,40-44].

Fullerene solubility

Fullerenes show poor solubility in aqueous and organic solvents. Once in contact with water, fullerenes form colloidal aggregates which are barriers to biomedical applications [45]. The advantage of aqueous solubility for fullerenes has spurred investigation into applications to modify fullerene delivery, such as chemical modification of the fullerene carbon cage, incorporation of fullerene into water-soluble micellar structures, solvent exchange, and long-term stirring in water [45].

Possible advantages of fullerenes

Research into the more complex NOLF molecule has been limited. However, their increasing use as bone and collagen scaffolds, drug and gene carriers, and biosensors are being explored with favorable results [46,47]. Also, NOLF has shown potential for the protection of neuronal circuitry and long-term memory [48]. Most observations and anecdotal reports of favorable clinical properties of C_{60} (and other single-walled fullerenes) are relevant for NOLF as well as other graphene-based nanocarbons. These shared effects suggest commonality in chemical or electrodynamic characteristics and properties among all graphene-based nanocarbons.

The enhanced antioxidant action of fullerenes corresponds directly to the increased size of the shell [49]. Be that as it may, the potential for significantly higher antioxidant capacity of the larger NOLF molecule has not been fully explored; nor has the impact resulting from shell defects often found in larger NOLF material.

Anionic fullerene derivatives have shown antioxidant properties; whereas, cationic derivatives have shown antibacterial and anti-proliferative activities. The amino acid type derivatives have been found to be the most active of all derivatives of fullerenes [50]. Enhanced longevity and diminished tumorigenesis using NOLF material were reported in the Baati study [11] and observed in the Desantis research [51]. An abridged list of biomedical research, based on classical concepts of physics and biology regarding useful applications of fullerenes, follows:

A. Anti-inflammatory action [9,52,53]

B. Anti-proliferative and anti-atherogenic effects [43]

C. Antimicrobial activity through growth inhibition [12,54]

D. Anti-virus and anti-HIV activity of fullerenes [22,42,55]; anti-hepatitis C [56] and anti-influenza virus [57]

E. (Apparent) antioxidant activity and free radical scavenging [9,40-42,57,58]; NrF2–induced cellular antioxidant defense [40,44]

F. Bioimaging in medicine [59,60]

G. Biosensors [47]

H. Cancer therapy [9,22,42,59,61-64]: antineoplastic activity [65,66]; and Caspase-independent autophagy-induced cancer cell death [21,67]

I. Drug delivery [9,22,61,68-70]

J. Electromagnetic field (EMF) and radiation protection [22]

K. Immune system effects [9,71,72]

L. Ischemia and reperfusion injury [43]

M. Neurodegeneration, neuronal protection, and stroke [9,22,42,43,73,74]

N. Osteoarthritis and intervertebral disk (IVD) degeneration [42,75]

O. Photodynamic therapy [22,43,76]

P. Skin [42]

Q. Stem cell research [46,77-79]

Reviews of the biocompatibility of fullerenes along with biomedical applications can be found in *Medicinal Applications of Fullerenes* [50] and the more recent *Fullerene: biomedical engineers get to revisit an old friend* [20]. Much of this research concerns the single-shell C_{60} and its functionalized variants.

Conclusion

Nanocarbon-based materials, despite evidence for concern, have demonstrated the potential for application in many areas of medicine. It is becoming more evident that toxicity and biocompatibility of nanocarbon-based materials, and NOLF itself, cannot be determined based on characterizations, properties, and in vitro testing alone. In vivo testing of individual fullerenes indicates that the low toxicity and proposed biocompatibility should outweigh generalizations regarding nanoparticle toxicity based solely on in vitro testing. Further research is needed to enhance the understanding of the biological interactions of fullerene-like materials to allow medical researchers to develop ways to utilize the tremendous potential they present. Fullerenes, in short, show promise; however, it is difficult for research to move forward swiftly (due primarily to the lack and consistency of pristine fullerene material) to develop a solid foundation of replicable research from which to explore and unlock the promising advantages and medical applications of fullerenes.

Conflict of Interest

The authors declare that this paper was written in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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