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Review Article

Electron Scavenging Not Free Radical Scavenging by Fullerene Materials Protects against Mitochondrial Oxidative Stress in Complex Organisms

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Abstract

Fullerene materials are often reported to be excellent antioxidants in cells. Usually, direct free radical scavenging mechanisms, as seen in polymer and food industry research, is the proposed antioxidant mechanism. However, free radical scavenging by antioxidants *in vivo* is kinetically unlikely and conflicts with the molecular properties of fullerene materials. Pristine fullerene materials, and many of their derivatives, have pro-oxidant pro-oxidant properties and form adducts, which are both potentially toxic mechanisms *in vivo*. Although they are electrophilic with an affinity for electrons, they appear to function similarly to other non-radical electrophilic antioxidants that stimulate enzymatic oxidative stress management and protection through Nrf-2 pathways. We approach the antioxidant role of fullerene materials from the perspective that they do not act as direct free radical scavengers. Other mechanisms have been previously proposed that are more consistent with fullerene properties and antioxidant benefits *in vivo*. Here we discuss fullerene materials as electron scavenging antioxidants rather than free radical scavengers. We propose that fullerene materials scavenge excess electrons in the electron transport chain, preventing the formation of superoxide and hydroxyl radicals in the mitochondria. The scavenging of electrons would assist in preventing damage to mitochondrial structures and DNA, maintaining optimum oxidized to reduced nicotinamide adenine dinucleotide (NAD+/NADH) ratios, as well as in maintaining an oxidized respiratory enzyme system in mitochondrial conditions resulting from excess caloric intake in the absence of cellular energy requirements and progressive mitochondrial dysfunction related to oxidative stress. Further research is needed to evaluate the utilization of fullerene materials for use in metabolic syndromes and neurodegenerative conditions that may be complicated by progressive mitochondrial oxidative damage.

Keywords: *C_{60}; Native Aggregation; Dielectric Property; Fullerene Material; Oxidative Stress; Mitochondria; NAD^+/NADH Ratio; Antioxidant; Electrophilic; Free Radical Scavenger; Electron Scavenger*

Abbreviations

ETC: Electron Transport Chain; OX/PHOS: Oxidation/Phosphorylation; REDOX: Reduction-Oxidation; NAD^+: Oxidized Nicotinamide Adenine Dinucleotide; NADH: Reduced Nicotinamide Adenine Dinucleotide; $NADP^+$: Oxidized Nicotinamide Adenine Dinucleotide Phosphate; NADPH: Reduced Nicotinamide Adenine Dinucleotide Phosphate; NAD(H) NAD^+/NADH Ratio: NADP(H): $NADP^+$/NADPH Ratio; Nrf-2: Nuclear Factor-Like 2; NADK: Nicotinamide Adenine Dinucleotide Kinase

Introduction

The accumulation of mitochondrial oxidative damage resulting in loss of redox homeostasis associated with many diseases, including neurodegeneration, cancer, and aging [1,2]. Electrons that leak from the electron transport chain (ETC) are the primary source of potential mitochondrial damaging reactive oxygen species (ROS) [3] and reactive nitrogen species RNS [4], which can further damage the mitochondria and decrease the efficiency of the ETC, perpetuating a feedback loop of ROS generation and subsequent mitochondrial damage when endogenous oxidative stress response becomes overwhelmed [5,6].

Exogenous antioxidants have been proposed to alleviate oxidative stress, and fullerene materials are often reported to be super antioxidants. Although fullerene materials have been shown to inactivate the hydroxyl radical by attachment to a carbon-carbon bond, structural properties may preclude fullerene materials from being super free radical scavengers *in vivo*. Pristine fullerenes are unique electrophilic molecules with a strong electron affinity. Electrophiles typically have a positive charge, but the degenerate lower orbital arrangement of fullerenes contains the unpaired electrons rather than in their outer shell. This arrangement results in low reactivity *in vivo*, yet like other electrophiles, they seek electrons. *In vitro*, they often behave like electron-deficient alkenes and primarily react through addition and substitution reactions with nucleophiles. The newly formed intermediate carbanion can react with electrophilic free radicals, which with it may come in contact. Through this proposed mechanism, they may become reduced electron donors and can functionally reduce or scavenge other free radicals, as has been suggested by *in vitro* and polymer studies [7]. Interestingly, evidence suggesting that a super free radical scavenging model for any exogenous antioxidant is unlikely a primary mechanism in living systems [7] and also strongly suggests that, at least for pristine fullerenes, they do not function in this manner. Pristine fullerene materials appear to be highly effective at reducing antioxidative stress, but their antioxidant activity is not synonymous with free radical scavenging in biological systems [8].

Given the prevailing concept of a free radical scavenger as an antioxidant, it may appear confusing that the free radical scavenging antioxidant activity used in polymer chemistry and food preservation, which enables the interplay of phenolic and hydroperoxide-reducing compounds and would not be efficient nor likely in biological systems. Free radical scavenging via electrophilic and nucleophilic reactions would be rate-limited, and substrate concentration-dependent rather than under cellular control, thus proceeding until one or the other substrate was depleted.

However, ROS production is not simply a toxic by-product of ATP production. When ATP production is highest, the production of ROS is low. Conversely, in hypoxic states, the production of ROS may be high. There are several electron donor sources in the mitochondria, so ROS production is a result of the concentration of potential electron donors, the local concentration of O_2 and the second-order rate constants for the reactions between them. Given a large number of potential electron donors within the mitochondria available to react with O_2 and the relatively few donors that do so suggests that in healthy cells, at least, the production of ROS is a highly regulated event [9]. Uncontrolled reactions, whether oxidative or reductive, in cells would be pathological and result in a breakdown of the quantum critical charge transport necessary for life. Enzymatic oxidative stress management that responds to the critical dynamic needs of the cell is the primary mechanism in complex biological systems [7]. Even the often proposed scavenging of hydroxyl radicals in biological systems was recognized as primarily a catalytic-like mechanism and could not be explained by the commonly assumed antiradical activity of C_{60} [10].

In a previous article, it was proposed that the primary antioxidant benefits of fullerene materials are through NrF2 mediated cell signaling, fullerene–protein/structure docking effects, and fullerene- cell water interactions, which may impact enzymatic oxidative stress management, enhances antioxidant response and mediates mitochondrial biogenesis [8]. The effects of fullerene material influences on the redox states of NAD(H) and NADP(H) pools should also be considered in the evaluation of their antioxidant activity. We propose that fullerene material docking with mitochondrial enzymes and membranes results in both the 'scavenging' of electrons leaking from the ETC, as well as influencing membrane proton currents to beneficially affect NAD(H) and NADP(H) pools and their ratios, which helps maintain mitochondrial redox homeostasis, energy metabolism, genetic transcription and cell signaling events.

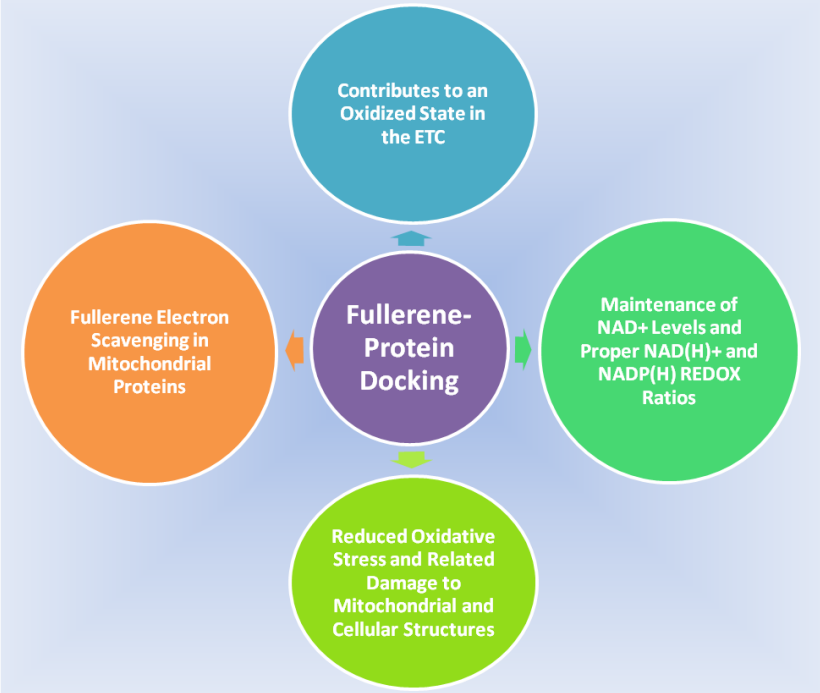

Figure 1: *Fullerene-protein docking effects.*

Fullerene-protein/membrane docking

Protein surfaces are highly receptive to fullerenes [11]. They are naturally amphiphilic and usually pH-responsive. Some researchers have expressed concern that fullerene-protein binding may result in protein inhibition and toxic effects [12]. Hydrophobic fullerene materials are well known to dock with proteins at polar and non-polar sites, and there were almost twice as many nonpolar than polar binding sites for C_{60} with proteins [13]. Among the nonpolar sites, π-π stacking arrangements with aromatic residues were important for stabilization of the fullerene-protein complex and were supported by cation-π and anion-π binding sites. Cation-π binding contributed to the stabilization of the α-helix. The delocalized electrons and double bonds in fullerenes seem to promote anion-π binding forces [14] as well as the weak hydrogen bond interaction with amino acid residues. The preferred amino acid residues for hydrophilic residues are GLU, LYS, ARG, ASN and ASP, in addition to the hydrophobic residues of ALA and LEU for fullerene interaction with proteins [13].

Proteins are being researched to stabilize and disperse small clusters of fullerenes in aqueous environments without the use of solvents, which may also control or decrease cytotoxicity [15]. It is noteworthy that the noncovalent protein interaction with fullerene materials, along with lipids and nucleic acids, is observed in the rapid formation of a biocorona that surrounds fullerene molecules. The fullerene biocorona may not only influence cellular uptake but also likely changes over time and in different microenvironments, both intra and extracellularly [16]. However, much remains unknown regarding biocoronas or the real effects of fullerene materials on protein structure and function or their interactions in different compartments of the cell. Research supports the enhancing effects of fullerenes on antioxidative enzymes [17] and it is very likely this extends to all active proteins in the cell, but it is not clear whether or not a fullerene-protein interaction or Nrf-2 mediated transcription, or both, increase.

Electron scavenging by fullerene materials

When oxygen utilization is low, and NADH continues to supply electrons to the ETC, the ETC is in a reduced state, and the protomotive force increases resulting in electron leak. In fact, in a reduced state, many reducing substrates in the ETC complexes can also directly contribute to superoxide production by donating electrons to O_2 [9]. As respiration slows, ROS production significantly increases [18,19]. When ROS generation is high during non-phosphorylating, rather than phosphorylating conditions, electron scavenging could effectively reduce oxidative stress and help maintain normal OX/PHOS respiration in the ETC.

Electron scavenging of electrons leaking from the ETC by fullerene materials located in or adjacent to the inner mitochondrial membrane could significantly relieve mitochondrial oxidative stress by preventing the formation of superoxide. In the ETC, electron carriers differ in their affinity for electrons, and electrons pass from a carrier with low affinity to a carrier with high affinity, and finally to O_2 as the last electron acceptor at Complex IV. Fullerene materials have a higher electron affinity than many biological molecules and most elements, including oxygen. The electron affinity for C_{60} (2.7 eV) is nearly twice that of monovalent oxygen (1.46 eV) or dioxygen (O_2) (0.450 eV), thus any escaping electron could be readily absorbed, or scavenged, by an available electron scavenger such as fullerene material. Complementary to mild uncoupling, which decreases phosphorylation in highly reduced ETC states, it is speculated that fullerenes within and adjacent to the inner mitochondrial membrane could act as an electron sink, thus acting to normalize respiration as well as reduce unnecessary energy expenditure. It should be noted that as fullerene size increases, so does their electron affinity [20] and interestingly, so does their potential decreased toxicity [21] and overall antioxidant activity [22,23]. Thus, NOLFs may emerge as the preferred electron scavenger and thereby offer a greater biologically responsive antioxidant activity than C_{60}.

NAD(H) and NADP(H) redox couples are essential for mitochondrial and cellular function, redox homeostasis, and mediation of cellular metabolism

NAD(H) and NADP(H) redox couples are critical for mitochondrial and cellular function, redox homeostasis and a deficiency or imbalances in these REDOX couples are associated with many pathological conditions [24]. The redox couple NAD(H) is primarily involved in the regulation of glycolysis and OXPHOS, while the NADP(H) couple is involved in maintaining redox balance and supporting the biosynthesis of fatty acids and nucleic acids. The mitochondrial membrane is considered impermeable to both NADH and NADPH, and both are regulated by multiple shuttles to maintain REDOX and energy homeostasis. This paper does not allow for a complete discussion of this topic, but Xiao., *et al*. present an excellent review of NAD(H) and NADP(H) redox couples and their involvement in cellular metabolism [2].

ROS production in mitochondria is facilitated by excess electrons present in mitochondrial proteins signifying their reduced state. NADH is the ETC's connection to upstream metabolism, including the Krebs cycle, delivering electrons to Complex I of the ETC where it is oxidized to NAD^+. ROS production by complex I requires a high NADH/NAD^+ ratio. Furthermore, Complex I can reverse the electron flow when the NADH/NAD^+ ratio is high, adding to ROS production. Complex III is another major source of ROS production whenever excess electrons are present. Hypoxia increases ROS production in most mammalian cells due to the failure of oxidation of both NADH and $FADH_2$, resulting in a build-up of reducing equivalents and reductive stress. The salvage pathway predominantly biosynthesizes NAD^+ and it is required, along with ATP, for the phosphorylation to $NADP^+$ via NAD kinase (NADK). $NADP^+$ is primarily reduced to NADPH by several enzymes in the cytosolic pentose phosphate pathway and within the mitochondria [2].

Fullerene materials within or docked with mitochondrial membranes or proteins may improve NAD(H) and NADP(H) REDOX couple ratios by electron scavenging. While NADPH is an electron donor in anabolic pathways, NAD^+ is an electron acceptor in catabolic energy production. Excess electrons delivered by NADH precipitate a reduced ETC resulting in electron leak and subsequent increased oxidative stress. It is not known whether or not fullerene materials participate directly as electron-accepting coenzymes in a manner similar to NAD^+ or $NADP^+$, but a recent toxicological assay paper using a bioluminescent assay of C_{60} based on inhibition of NADPH oxidoreductase

oxidation to $NADP^+$ demonstrated little inhibition or enhancement [25]. However, electron scavenging helping to prevent a reduced state in the respiratory enzymes would help maintain elevated NAD^+/NADH ratios, along with manageable levels of oxidative stress.

Fullerene materials may improve NAD(H) and NADP(H) redox couples

Fullerenes are not only biocompatible but appear to have diverse benefits that often reported by self-experimenters using NOLF [26]. Limited research such as Baati., *et al.* report enhanced longevity, along with the retention of more youthful characteristics in energy and appearance [27].

Desantis, regarding his experience after the NAMT treatment [28] with the mice injected with a bolus of NOLF, revealed similar observations, including one mouse that only received the NOLF injection. These observations and reported effects are not easily explained by antioxidant activity alone. However, NAD(H)and NADP(H) REDOX couple pools drop precipitately during aging and are often associated with disease states. Declining NAD^+ levels may be a central mechanism that connects the diseases of aging and even cancer. Restoring and maintaining the NAD^+ pool along with a high NAD^+/NADH ratio with pharmacologic or nutrient-based supplementation appears to help in maintaining critical cell processes dependent upon NAD^+ such as the activities of sirtuins, poly-ADP-ribose polymerases (PARPs) and CD38/157 ectoenzymes, as well as gene repair, metabolism, and the circadian rhythm during aging and degenerative diseases [29-31].

Conclusion

The use of various NAD^+ precursors has already been suggested as a therapeutic intervention in metabolic and neurodegenerative diseases. Fullerene materials would appear to offer tremendous benefit for both antioxidant activity as well as increasing NAD^+ and promoting favorable NAD^+/NADH ratios in healthy and dysfunctional cells. However, the proposal that fullerene materials scavenge electrons in the respiratory enzyme pathways and increase NAD^+ and NAD^+/NADH ratios require confirmation and further research to determine optimum therapeutic parameters.

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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