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Hydrophilic carbon clusters as therapeutic, high capacity antioxidants

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Abstract

Oxidative stress reflects an excessive accumulation of reactive oxygen species (ROS) and is a hallmark of several acute and chronic human pathologies. While many antioxidants have been investigated, the majority have demonstrated poor efficacy in clinical trials. Here, we discuss limitations of current antioxidants and describe a new class of nanoparticle antioxidants, poly(ethylene glycol)-functionalized hydrophilic carbon clusters (PEG-HCCs). PEG-HCCs show high capacity to annihilate ROS such as superoxide and hydroxyl radicals, show no reactivity toward nitric oxide, and can be functionalized with targeting moieties without loss of activity. Given these properties, we propose that PEG-HCCs offer an exciting new area of study for treatment of numerous ROS-induced human pathologies.

Keywords

antioxidant; traumatic brain injury; carbon nanoparticle; oxidative stress

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Oxidative Stress as a Therapeutic Target

Oxidative stress is a state in which the equilibrium of pro-oxidants and antioxidants shifts in favor of pro-oxidant species. ROS contain unpaired electrons that are highly reactive toward other molecules such as nucleic acids, lipids and proteins. Oxidative damage to nucleic acids can lead to modifications of genetic material that contribute to mutagenesis. Lipid peroxidation is the reaction of ROS and lipids in a free-radical chain sequence also known as autoxidation. Oxidative damage to proteins can lead to alterations in their primary, secondary, and tertiary structure and of enzymes, leading to inactivation [1].

Superoxide ($O_2^{\bullet -}$) is a radical anion that is considered a primary ROS and it can form secondary ROS through interaction with other molecules, metals, or enzymes [2,3]. For example, $O_2^{\bullet -}$ can facilitate production of the reactive hydroxyl radical ($HO^{\bullet}$) by releasing iron from iron-sulfur containing enzymes [4]. In addition, $O_2^{\bullet -}$ can lead to the generation of hydrogen peroxide (H_2O_2) through dismutation. In the presence of nitric oxide ($NO^{\bullet}$), a radical used by several tissues as a signaling molecule, the highly reactive anion peroxynitrite ($ONOO^-$) is formed. This species is implicated in lipid peroxidation and oxidative damage [5]. Lipid oxidation generates lipid free radicals (•R).

Shortcomings of classical antioxidants

Despite the plethora of data on oxidative stress in disease, including acute ischemic injury, most large clinical trials with antioxidants have shown little to no benefit in disease treatment [6]. We propose that the critical limitations of currently available antioxidants (Table 1) include one or more of the following: (i) requirement for the presence of additional downstream enzyme(s) to detoxify the radical product of an upstream molecule, (ii) limited number of radicals removed per antioxidant moiety, (iii) antioxidant regeneration by enzymes which may be consumed in the toxic milieu, and (iv) production of additional radicals through the antioxidant's mechanism of action.

The *in vivo* response to oxidative stress is to transfer the free radical through a chain-reaction that requires the concerted action of many antioxidants. For instance, superoxide dismutase and catalase work sequentially to convert $O_2^{\bullet -}$ to H_2O_2 and oxygen and then finally to oxygen and water [7,8]. As a result, this arrangement necessitates that both enzymes are present to effectively destroy the radical. Furthermore, the majority of classical antioxidants can remove at most two radicals per molecule of antioxidant. Most antioxidants such as glutathione, vitamin C, and vitamin E reduce an ROS by donating electrons to the free radical, and in the process generating an additional ROS (oxidized vitamin C and vitamin E) which then requires additional enzymes to regenerate the antioxidant. These limitations may provide one explanation why, even though transgenic models that overexpress antioxidants show quite robust protection against acute injury, there is little evidence for benefit of antioxidant therapy in a clinical setting when therapy begins following the injury [9,10].

In cases of high oxidative stress, such as those following acute nervous system injuries, in conditions of ischemia or reperfusion, or during hemorrhagic shock and resuscitation, free radicals may be transferred to nearby proteins, nucleic acids or lipids, which could lead to

additional biological damage. Classical antioxidant systems would be exhausted under such conditions, and regeneration of antioxidants might not occur. Therefore, antioxidants that can quench or dismutate multiple radical species or act as terminal acceptors to a large number of ROS might be more beneficial during conditions of excessive oxidative bursts than antioxidants that require regeneration.

Nano-antioxidants

The need for efficient antioxidants has led to the development of nanoparticle antioxidants, which can include many structures such as liposomes and metal. Metal oxide nanoparticles such as cerium oxide (CeO_2) and yttrium oxide (Y_2O_3) have shown promising results in several disease models [11]. Other precious metals such as gold or platinum stabilized with pectin were able to quench $O_2^{\bullet -}$ and H_2O_2 [12]. In a similar vein, nano-jewels, composed of a diamond nanoparticle scaffold supporting either gold or platinum nanoparticles, have nearly 2-fold higher antioxidant activity than glutathione [13].

Carboxy-functionalized carbon-based buckminsterfullerenes (C_{60}) were found to be highly reactive with ROS, and this may be mediated through their highly conjugated double bond system [14]. Several water-soluble derivatives of C_{60} were synthesized and tested in cells and found to be neuroprotective in cultured cortical neurons [15]. C_{60} and its derivatives have been reported to react with $O_2^{\bullet -}$ radicals, hydroxyl radical, alkylperoxyl radicals, alkoxyl radicals, and benzyl radicals [15–17]. Some C_{60} derivatives were found to possess SOD mimetic properties, although their rate constant was around 100-fold slower than SOD [16] and functionalization reduced activity in some circumstances. [14] Carbon nanotubes have also been found to have antioxidant activity [18,19]. However, there are concerns regarding toxicity of these structures [20].

Poly(ethylene glycol)-functionalized hydrophilic carbon clusters (PEG-HCCs): active nanovectors

The limited aqueous solubility of many new therapeutics and promising drug candidates is a long-standing challenge in the pharmaceutical industry. A safe, modular drug delivery platform consisting of small (<40 nm) poly(ethylene glycol)-functionalized hydrophilic carbon clusters (PEG-HCCs) is a possible solution [21]. Because of the presence of hydrophobic domains on the HCC core, PEG-HCCs would be excellent carriers for hydrophobic drugs such as paclitaxel, docetaxel, SN-38, prednisone, rosiglitazone, idarubicin, vinblastine, and glibenclamide. Drugs can be loaded non-covalently and the resulting aqueous solutions are stable at room temperature for at least 5 months [21].

This platform was extended into a targeted drug delivery vehicle by functionalizing the PEG-HCCs with both monoclonal antibodies and targeting peptides. PEG-HCCs can be noncovalently functionalized with the antibody to the epidermal growth factor receptor (EGFR) (Cetuximab (Cet)) for specific delivery of unmodified PTX to $EGFR^+$ tumors but not $EGFR^-$ tumors [22,23]. Analogous delivery to tissue specific targets in glioblastoma multiforme (GBM), an aggressive human brain cancer with poor clinical outcome, has

shown enhanced tumoricidal activity in multiple cell lines without evidence of toxicity to normal human astrocytes [24].

Subsequently, it was thought that the graphitic structure of the HCC core would result in antioxidant activity, as has been shown for fullerene derivatives [13–15]. Indeed, PEG-HCCs have been shown to be remarkable antioxidants, and are able to annihilate ROS such as superoxide and hydroxyl radical *in vitro* and *in vivo* [25,26]. As such, PEG-HCCs offer an exciting new area of study for treatment of numerous pathologies in which ROS are implicated, and could potentially succeed where classical antioxidants have failed.

PEG-HCCs are antioxidants

PEG-HCCs possess a remarkable ability to quench $O_2^{\bullet -}$ and $HO^{\bullet}$ while being inert to NO and $ONOO^-$ [25]. One potential mechanism for quenching is the catalytic dismutation of $O_2^{\bullet -}$ whereby the HCC core of the PEG-HCC is not destroyed:

$$\mathrm{PEG{-}HCC^{\bullet} + O_2^{\bullet -} \rightarrow PEG{-}HCC^{-} + O_2} \quad (1)$$

$$\mathrm{PEG{-}HCC^{-} + O_2^{\bullet -} + 2\,H_2O \rightarrow PEG{-}HCC^{\bullet} + H_2O_2 + 2\,HO^{-}} \quad (2)$$

This proposed mechanism of action, while similar to a previously described tris-malonic acid derivative of C_{60} (C_3), does not invoke the same high entropy transition state [16]. A PEG-HCC can accept an electron from $O_2^{\bullet -}$ (eq 1) to form a highly delocalized electron pair on the conjugated carbon core, followed by the donation of an electron to a second molecule of $O_2^{\bullet -}$ (eq 2) accompanied by the rapid capture of two protons from water to complete the catalytic cycle.

PEG-HCCs are readily internalized by murine brain endothelial (bEnd.3) cells and were effective at reducing ROS levels when administered 10 minutes after induction of oxidative stress, whereas PEG-SOD and PBN, a small molecule antioxidant, required pretreatment with 10-fold or higher dose to achieve comparable ROS reduction [26]. Moreover, treatment with PEG-HCCs significantly restored cell viability whereas PEG-SOD and PBN displayed minimal cellular protective effect when administered following the onset of oxidative stress [25,26].

Synthesis, toxicity, and biodistribution of PEG-HCCs

HCCs are prepared by subjecting single-walled carbon nanotubes (SWCNTs) to a harsh oxidation procedure that uses a mixture of fuming sulfuric acid and nitric acid [21]. Careful control of temperature and time yields a carbon material which bears very little resemblance to the starting SWCNTs. The surface of the HCCs harbors a variety of oxygen-containing moieties such as alcohols, ketones and carboxylic acids. To increase water solubility, poly(ethylene glycol) (PEG) is covalently attached to the HCCs via standard carbodiimide-coupling chemistry. PEG-HCCs have a hydrodynamic diameter of 35 to 40 nm, which is comparable to a medium-sized protein. The final product is a nanoparticle that can be further functionalized and used in a variety of applications (Figure 1) [27].

As with any newly designed therapeutic compound, toxicity considerations are of utmost importance. Carbon nanotubes have been suggested to be hazardous due to their superficial resemblance to asbestos [20]. Although derived from SWCNTs, PEG-HCCs are highly functionalized, well dispersed, hydrophilic, small in size, and contain little to no contamination with metals. Thus, they bear no resemblance to asbestos, and these characteristics should allay fears of toxicity [28–30]. Mice treated with PEG-HCCs weekly for 10 weeks showed no signs of discomfort, fatigue, or weight loss; histology revealed no toxicity to heart, lungs, spleen, kidneys, liver, or brain; renal and liver markers were unchanged and hematology was normal [21]. PEG-HCCs accumulated mostly in the spleen, liver, and kidneys, were excreted through the urine, and their blood half-life was estimated to be about 2 to 3 hours [21]. Although PEG-HCCs did not accumulate in large quantities in the brain parenchyma, functionalizing them with a lipophilic moiety such as adamantane [31] might enable biodistribution to the brain.

While differing in nearly all major features from carbon nanotubes, the conjugated carbon framework may make it possible for PEG-HCCs to be eliminated in the same fashion as smaller carboxylated or oxidized carbon nanotubes, which can be degraded by enzymes such as horseradish peroxidase [32] and myeloperoxidase [33] in the presence of radicals such as H_2O_2 or by conditions created to simulate the phagolysosomal environment.

PEG-HCCs as Potential Therapeutic Agents in Experimental Models

Traumatic brain injury is a bi-phasic injury in which the first phase is the mechanical damage to the brain and the second phase is the activation of various biochemical, physiological, and molecular cascades that lead to exacerbation of the initial insult. Not only are ROS produced immediately after the primary mechanical injury to the brain, but also during secondary events initiated by TBI and during treatment such as resuscitation and reperfusion of blood that had been lost by a secondary insult [34,35]. Secondary insults such as significant blood loss (hemorrhagic shock), hypotension or hypoxia significantly worsen outcomes in animal models of TBI and are associated with increased mortality in TBI patients [36]. There are currently no FDA-approved treatments proven to mitigate the effects of TBI.

Many therapeutic agents have entered clinical trials for TBI and, unfortunately, the majority of them showed no significant improvement in patient outcome and survival, other than perhaps some specific subgroups. Because ROS production is correlated with the second phase of damage in TBI [35], it is possible that the damage could be due to oxidative stress, thus antioxidants have been a major point for consideration. The antioxidant compounds PEG-SOD and tirilazad, an inhibitor of lipid peroxidation, have both been tested in multicenter phase III trials with no significant benefit in the overall TBI population, although post hoc analysis demonstrated potential benefit in certain subsets of patients [34,37,38]. In a small study, vitamin C and vitamin E each had mixed results [39]. Dexanabinol, a mixed glutamate antagonist and antioxidant drug, also failed to show therapeutic benefit in a randomized, placebo-controlled clinical trial in severe TBI [40].

Given the great potential of PEG-HCCs to remove $O_2^{\bullet-}$, we postulate that these carbon nanoparticles could inhibit oxidative damage and possibly restore autoregulation in experimental TBI models. Indeed, PEG-HCCs restored cerebral blood flow in rats to pre-injury levels temporarily (consistent with their blood half-life) and reduced $O_2^{\bullet-}$ levels compared to vehicle-treated animals [26]. With this result, it is conceivable that native or targeted PEG-HCCs might be therapeutically useful in other cases, such as for acute and chronic diseases, including: nonalcoholic fatty liver disease (NAFLD), chemotherapy induced neurotoxicity, multiple sclerosis, and rheumatoid arthritis, as oxidative stress has been implicated in each [41,42].

Concluding remarks and future perspectives

The body is inevitably subjected to oxidative stress and, if not adequately resolved, various resulting pathologies could culminate in disease. Although traditional antioxidants would seem to offer a remedy, their success has been limited, possibly due to their mechanism of action. Most antioxidants possess low radical capacity (i.e. they can detoxify 1 to 2 radicals per molecule of antioxidant), and must depend on other enzymes to complete the detoxification and regenerate the antioxidant. PEG-HCCs are unique due to their high antioxidant capacity, the possibility of catalytic behavior, and non-reliance on detoxifying enzymes. Additional advantages include the potential to become targetable via simple mixing with antibodies and carry additional drug payloads. PEG-HCCs thus offer an exciting new area of study for treatment of numerous ROS-induced human pathologies.

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Box 1

Outstanding Questions

- What features of PEG-HCC structure are responsible for the catalytic antioxidant activity and the preferred reaction produces, e.g. oxygen vs hydrogen peroxide?
- To what extent are these antioxidant properties generic to oxidized carbon nanoparticles?
- What is the *in-vivo* duration of action?
- Is the metabolic fate of PEG-HCCs similar to other carbon nanomaterials in relation to breakdown by endogenous peroxidases?

Highlights

- Most antioxidants show little efficacy following trauma such as brain injury or stroke.
- Carbon nanoparticles quench superoxide affording no downstream radicals.
- Each nontoxic nanoparticle can annihilate thousands of superoxide molecules; the same nanoparticles are selective, being inert to nitric oxide.
- Prospects are shown for treatment of numerous superoxide-induced human pathologies.

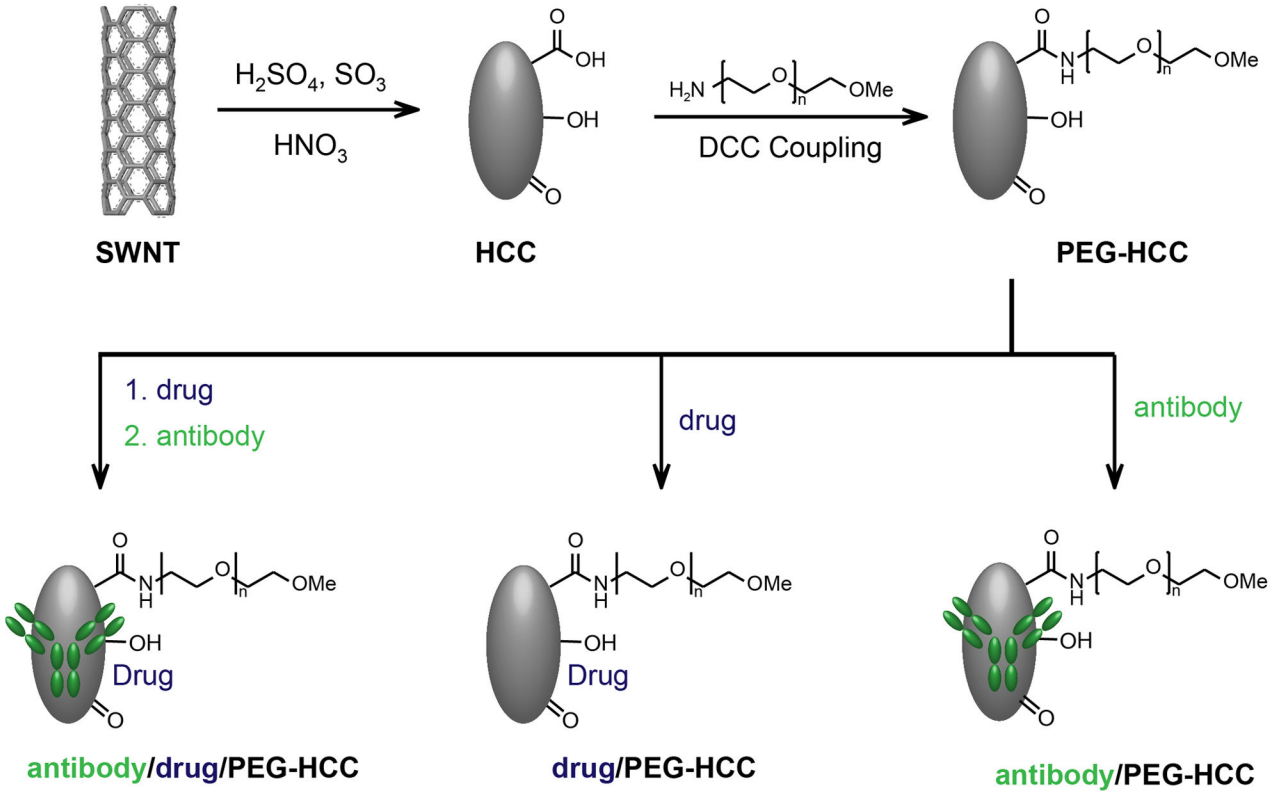

Figure 1. HCC synthesis and functionalization

HCCs are prepared from SWNTs by oxidation in fuming sulfuric acid and nitric acid (top left). The carboxylic acids on the resulting HCCs are coupled to PEG to form PEG-HCCs (top right), which can be used to non-covalently sequester hydrophobic drugs, targeting antibodies or both for a variety of medicinal applications (bottom).

Table 1

Mechanism of action of various antioxidants

Antioxidant	Target ROS	# of ROS removed per molecule	ROS generated	Detoxifying enzyme
SOD	$\bullet O_2^-$	2	H_2O_2	none
catalase	H_2O_2	2	none	none
glutathione peroxidase	H_2O_2	1	none	none
glutathione	H_2O_2	1	none	glutathione reductase
vitamins E	$\bullet O_2^-$, •R	1	•E	vitamin C
vitamin C	•E, •R	1	•C	dehydroascorbate reductase
albumin	•OH	not known	none (presumably by disulfide formation)	none
PBN	$\bullet O_2^-$, •OH	1	nitroxide free radical	none
tempol	$\bullet O_2^-$, ONOO	1	H_2O_2, •NO	none
fullerene derivative (C_{60})	$\bullet O_2^-$, •OH	not known	none	none
PEG-HCCs	$\bullet O_2^-$, •OH	estimated 10^6	H_2O_2	none