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Hydrophilic carbon clusters as therapeutic, high-capacity antioxidants

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Oxidative stress reflects an excessive accumulation of reactive oxygen species (ROS) and is a hallmark of several acute and chronic human pathologies. Although many antioxidants have been investigated, most have demonstrated poor efficacy in clinical trials. Here we discuss the 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 ($O_2^{\bullet -}$) and the hydroxyl ($HO^{\bullet}$) radical, show no reactivity toward the nitric oxide radical ($NO^{\bullet}$), 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 the treatment of numerous ROS-induced human pathologies.

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].

$O_2^{\bullet -}$ is a radical anion that is considered a primary ROS and 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 $HO^{\bullet}$ 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 $NO^{\bullet}$, which is 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 ($R^{\bullet}$).

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 enzymes to detoxify the radical product of an upstream molecule; (ii) limited number of radicals removed per antioxidant moiety; (iii) antioxidant regeneration by enzymes that 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 (SOD) and catalase work sequentially to convert $O_2^{\bullet -}$ to H_2O_2 and oxygen and then finally to oxygen and water [7,8]. This arrangement necessitates that both enzymes are present to effectively destroy the radical. Furthermore, most classical antioxidants can remove at most two radicals per molecule of antioxidant. Most antioxidants, such as glutathione, vitamin C, and vitamin E, reduce ROS by donating electrons to the free radical and in the process generate an additional ROS (oxidized vitamin C or vitamin E) which then requires additional enzymes to regenerate the antioxidant. These limitations may provide one explanation why, although transgenic models that overexpress antioxidants show quite robust protection against acute injury, there is little evidence for benefit of antioxidant therapy in clinical settings when therapy begins following the injury [9,10].

In cases of high oxidative stress, such as those following acute nervous system injuries, under conditions of

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Table 1. Mechanism of action of various antioxidants

Antioxidant	Target ROS	Number of ROS removed per molecule	ROS generated	Detoxifying enzyme
SOD	$O_2^{\bullet-}$	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
Vitamin E	$O_2^{\bullet-}$, $R^{\bullet}$	1	$E^{\bullet}$	Vitamin C
Vitamin C	$E^{\bullet}$, $R^{\bullet}$	1	$C^{\bullet}$	Dehydroascorbate reductase
Albumin	$HO^{\bullet}$	Unknown	None (presumably by disulfide formation)	None
PBN	$O_2^{\bullet-}$, $HO^{\bullet}$	1	Nitroxide free radical	None
Tempol	$O_2^{\bullet-}$, $ONOO^-$	1	H_2O_2, $NO^{\bullet}$	None
Fullerene derivative (C_{60})	$O_2^{\bullet-}$, $HO^{\bullet}$	Unknown	None	None
PEG-HCCs	$O_2^{\bullet-}$, $HO^{\bullet}$	Estimated 10^6	H_2O_2	None

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 for a large number of ROS might be more beneficial under 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]. Similarly, nano-jewels, comprising a diamond nanoparticle scaffold supporting either gold or platinum nanoparticles, have nearly twofold 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 radicals, 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].

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 comprising small (<40 nm) 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 (PTX), docetaxel, SN-38, prednisone, rosiglitazone, idarubicin, vinblastine, and glibenclamide. Drugs can be loaded noncovalently 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 $O_2^{\bullet-}$ and $HO^{\bullet}$ *in vitro* and *in vivo* [25,26]. Thus, PEG-HCCs offer an exciting new area of study for the 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:

$$\text{PEG-HCC}^{\bullet} + O_2^{\bullet-} \rightarrow \text{PEG-HCC}^- + O_2 \quad [1]$$

$$\text{PEG-HCC}^- + O_2^{\bullet-} + 2H_2O \rightarrow \text{PEG-HCC}^{\bullet} + H_2O_2 + 2HO^- \quad [2]$$

This proposed mechanism of action, although 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 $O_2^{\bullet-}$ (Equation 1) to form a highly delocalized electron pair on the conjugated carbon core, followed by the donation of