A New Class of Antioxidant Carbon Nanoparticles: Superoxide to Oxygen Generators

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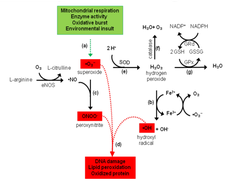
Abstract

Our primary defense against excess oxidative radicals consists of enzymes and proteins that generate toxic intermediates and themselves are consumed after injury. Antioxidant therapy has not been effective in clinical trials. We hypothesized these failures were due to limitations of current antioxidants including low capacity, requirement for regeneration, limited range and generation of toxic intermediates. We developed a new class of antioxidant based on highly modified, 40 nm long carbon nanoparticles termed PEGylated hydrophilic carbon clusters (PEG-HCCs). We previously reported rapid cellular uptake and complete restoration of neurovascular unit dysfunction in a model of traumatic brain injury and hypotension/resuscitation. Here we explored their mechanism and in-vitro effectiveness. Electron paramagnetic resonance (EPR) studies demonstrated high capacity for catalytic transformation of superoxide to oxygen while quenching hydroxyl radical. In-vitro, they protected to within 80-90% of baseline cell count when administered AFTER the mitochondrial toxin, Antimycin A, or direct application of hydrogen peroxide in brain endothelial or primary neuronal cell culture. The uniquely favorable mechanisms of these materials suggest their potential ability to overcome limitations of current antioxidants in ischemia/reperfusion.

Background

Based on many lines of evidence, oxidative stress is a major pathophysiological factor in stroke. This evidence is exemplified by robust protection in multiple transgenic antioxidant overexpression models of ischemia/reperfusion. However, treatment *following* injury at relevant time points is not consistently beneficial and no clinical trial of antioxidant therapy in any form of brain injury has shown benefit. We believe these failures are due limitations in currently available antioxidants that hinder their effectiveness *following* injury. Several defense mechanisms exist to cope with oxidative radicals generated during normal physiology (see Figure below). These mechanisms consist of enzymes and other proteins that modify the radical species in a series of steps ultimately leading to water. In the case of superoxide radical (SO), intermediate unstable molecules (e.g. hydrogen peroxide; H_2O_2) or new radicals (hydroxyl; OH) are generated by this process. Under normal conditions there are sufficient levels of protective proteins for detoxification. However, under pathological circumstances, these protective factors are depleted. After acute injury, these cannot upregulate fast enough. As a result, unstable intermediates are formed that become part of a radical cascade leading to damage and disruption of a wide variety of vital functions. We can summarize the limitations of current antioxidants that include one or more of the following: a mechanism of action in which the radical is transferred to another unstable species, exemplified by superoxide dismutase (SOD); the need for regeneration, such as in vitamins E and C that require glutathione, itself consumed in the oxidative stress milieu; limited capacity that is inadequate to cope with the cascade of radicals following injury; and selectivity in which an agent is effective against only one radical type.



Endogenous antioxidant defense mechanisms that result in production of unstable intermediates and depend crucially on the presence of detoxifying enzymes and proteins. During injury, these are also consumed and require regeneration. Loss of downstream protection perpetuate a cascade of injury.

Soon after the discovery of carbon based buckminsterfullerenes (C_{60}), these materials were shown to have antioxidant characteristics. Subsequent modifications and applications to models of injury identified neuroprotective properties but also a low threshold for further modification lest their antioxidant capacity be reduced. Hydrophilic carbon clusters (HCCs; Fig.2) are highly effective antioxidants. These particles are small (40 nm in length, 1-2 nm in diameter, comparable to a hydrated protein), highly functionalized to generate hydrophilic moieties with the addition of poly(ethylene glycol) (PEG) to provide solubility in biological fluids, stable at room temperature and without apparent toxicity seen thus far. These hydrophilic sites also provide the opportunity to attach hydrophobic small molecules, short peptides and proteins including antibodies to facilitate targeting or alter their distribution in-vivo.

Figure 3 shows their ability to quench reactive oxygen species (ROS) released due to the mitochondrial toxin, Antimycin A as reflected in dihydroethidine fluorescence (DHE) in cultured b.End3 brain endothelial cell line compared to superoxide dismutase and the small molecule phenyl butyl nitrone (PBN), a precursor to the failed antioxidant NXY 925 (SAINT trial). These conventional antioxidants required pre-treatment to reach the same level of protection as PEG-HCCs administered 10 minutes **after** the Antimycin A. PEG-HCCs were effective in preventing cell death even after direct application of Hydrogen Peroxide (Fig. 4). We recently reported their ability to rapidly and completely restore cerebrovascular dysregulation in a rat model of mild traumatic brain injury followed by hemorrhagic hypotension and resuscitation (ACS Nano 2011; Figure 5.)

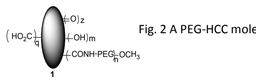


Fig. 2 A PEG-HCC molecule with estimated 2nm X 40 nM size and functional groups

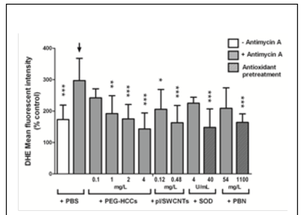


Fig. 3. ROS Quenching in cultured b.End3 cells after stimluation with Antimycin A. PEG-HCCs demonstrated a dose-dependent normalization of dihydroethidine (DHE) fluorescence when treated 10 min AFTER AntA, while SOD and PBN, prototype antioxidants, required 12 h pre-treatment and higher concentrations for comparable effect.

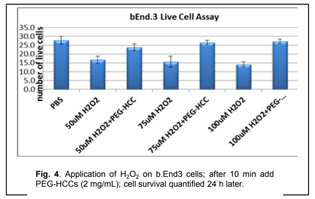


Fig. 4. Application of H_2O_2 on b.End3 cells; after 10 min add PEG-HCCs (2 mg/mL), cell survival quantified 24 h later.

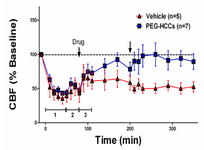


Fig. 5. PEG-HCC treatment improves cerebral blood flow (CBF) in the injured cortex in rats with TBI plus hemorrhagic shock (HS). CBF in the injured cortex is dramatically reduced following mild TBI and hypotension, and does not return to baseline in the vehicle-treated rats. Two sequential doses of PEG-HCCs, beginning at the time of definitive resuscitation (arrows) and repeated at 2 h completely restored CBF to baseline, with effects beginning within min of injection (top, injured cortex Phase 1 = TBI/HS; Phase 2 = saline infusion; Phase 3 = blood reinfusion with vehicle or PEG-HCC.

Objective

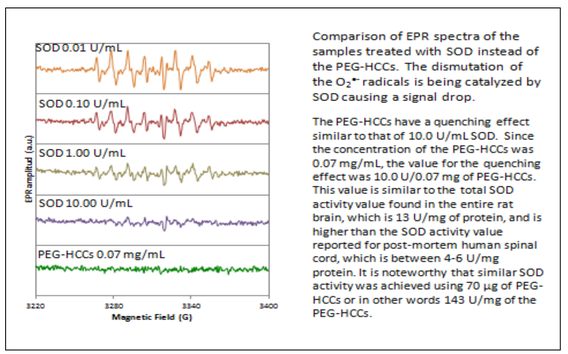
The objective of this study was to assess the mechanism of action of PEG-HCCs in explaining their powerful in-vitro and in-vivo protective actions against oxidative stress. We tested their antioxidant mechanism and their products following the interaction with superoxide through electron proton resonance (EPR) spectroscopy.

Methods & Results

The OH, SO, NO and peroxynitrite anion ($ONOO^-$) scavenging properties of PEG-HCCs using EPR spectroscopy, oxy-hemoglobin, cytochrome c, and pyrogallol red decomposition assays.

Quantitative electron paramagnetic resonance (EPR) indicated the quenching effect of PEG-HCCs is equivalent to the total SOD activity in human spinal cord (Figure below). Turnover numbers (moles of consumed SO moles of PEG-HCCs) were a dramatic ~1.3 million at 87,000s^{-1}. Nanomolar concentrations of PEG-HCCs (4 nM) showed typical Michaelis-Menten kinetics (Figure 7a). **The catalytic turnover number is about an order of magnitude higher than most efficient single active site enzymes,** and suggests that a PEG-HCC could possess multiple catalytically active sites. Furthermore, 2.4 nM of PEG-HCCs are able to scavenge 2.8 µM and 53.7 µM of **SO** and of **OH**, respectively. Finally, we found both through EPR and confirmed with Clarke electrode, the production of O_2 in equivalent kinetics to SO decay (Figure 7b).

Confirming our previous in-vitro and in-vivo studies, we found that PEG-HCCs are not quenching NO radicals (data not shown). PEG-HCCs had no effect on $ONOO^-$ (data not shown). Given that NO is constantly produced in- vivo, is freely diffusible and PEG-HCCs efficiently scavenge SO, this upstream scavenging effect will likely also decrease the amount of $ONOO^-$ produced in-vivo. **Taken together, these studies demonstrate that PEG-HCCs address each of the hypothesized limitations of current antioxidants.**



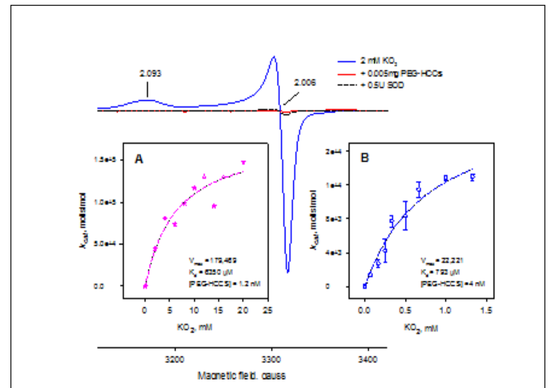


Figure 7. Direct EPR detection of O_2^- and PEG-HCCs catalyzed saturation kinetics of O_2^- decay (A) and O_2 production (B). The saturable kinetics of superoxide quenching and oxygen production are in virtually 1:1 stoichiometry indicating the likelihood that PEG-HCCs are directly converting superoxide anion to oxygen. Additional experiments indicated minimal detection of intermediate hydrogen peroxide, further confirming that PEG-HCCs are not acting as SOD mimetics, but act through a different pathway to quench the superoxide radical.

Discussion

We have developed a new class of carbon nano-particle based antioxidant we term polyethylene glycol-functionalized hydrophilic carbon clusters (PEG-HCCs) that overcome many limitations of current antioxidants. Here we demonstrate they possess the highly favorable characteristic of generating O_2 in a 1:1 stoichiometry with SO consumption while efficiently quenching multiple ROS. That profile exemplifies an ideal situation in an ischemic environment where the radicals would simultaneously be quenched while undergoing reoxygenation. **Hence, we term these materials as "superoxide to oxygen generators" (SOGs).**

References

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