Supporting Information

Samuel et al. 10.1073/pnas.1417047112

SI Materials

DEPMPO was obtained from Santa Cruz Labs. Amplex Red was purchased from Life Technologies. Ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), cupric sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), nitric acid (HNO_3), H_2O_2 (30% in water), potassium phosphate monobasic (KH_2PO_4), potassium permanganate ($KMnO_4$), ethanol (EtOH), sulfuric acid (H_2SO_4), and sodium hydroxide (NaOH) were purchased from Fisher Scientific. Potassium phosphate dibasic (K_2HPO_4) was purchased from J. T. Baker. Methoxypolyethylene glycol amine [PEG, molecular weight (MW) 5,000], Chelex, EDTA tetrasodium salt hydrate (EDTA), catalase from bovine liver, triethanolamine (TEA), methylsulfoxide (DMSO), sodium perchlorate ($NaClO_4$), HRP, deferoxamine mesylate (DFO), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), pyrogallol, sodium nitrite ($NaNO_2$), ascorbic acid (acid form), caffeic acid, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), hypoxanthine (HX), cytochrome *c* from equine heart, xanthine oxidase (XO), SOD from bovine erythrocytes (EC 1.15.1.1), CAPS, CHES, TAPS, and Hepes were purchased from Sigma-Aldrich. Crown ether (18-crown-6) was purchased from Fluka. Potassium superoxide (KO_2) was obtained from Acros Organics. The inductively coupled plasma standard for iron and the dialysis membrane [molecular weight cutoff (MWCO) 1,000 and MWCO 5,000] were purchased from High Purity standards and Biotech, respectively. Human oxyhemoglobin (HbO_2) was a gift from J. S. Olson, Rice University, Houston. C_{60}-serinol was donated by Lon Wilson, Rice University, Houston, and C_3 was donated by Douglas DeWitt, The University of Texas Medical Branch, Galveston, TX.

SI Methods

PEG-HCCs. HCCs were prepared by oxidizing single-walled carbon nanotubes using a mixture of oleum and nitric acid. These HCCs were then PEGylated using 5,000-MW α-amino-ω-methoxy PEG via standard carbodiimide coupling chemistry (1). The concentration of PEG-HCCs in solution is estimated based on the carbon core absorbance at 763 nm using an extinction coefficient of 0.01040 L/mg or 0.00428 nM^{-1} (1). The MW of the PEG-HCCs (411,509 g/mol) was estimated based on weight loss percentages obtained from thermogravimetric plots of the HCCs and PEG-HCCs. See Fig. S8 for calculations.

Oxyhemoglobin NO• Scavenging Assay. Oxyhemoglobin (HbO_2, 8 μM based on heme content, ε_{425nm} = 125 $mM^{-1}{\cdot}cm^{-1}$) was dissolved in TEA buffer (50 mM, pH 7.5). The NO• solution was prepared by bubbling NaOH trap-purified 99.9% pure NO• gas (Matheson) through 10 mL of anaerobic TEA (50 mM, pH 7.5) for 10 min. An ∼2 mM solution of NO• was obtained at atmospheric pressure and kept in a glass tonometer (2). The HbO_2 and the PEG-HCCs were mixed in the quartz cuvette before initiating the reaction with the addition of NO•. The final metHb concentration was 9.7 ± 0.3 μM ($\varepsilon_{401–411nm}$ = 38 $mM^{-1}{\cdot}cm^{-1}$) (3), and 0.02 mg/mL of both PEG-HCCs (48.6 nM) and PEG (4000 nM). The UV spectra were measured using a Hewlett-Packard 8453 diode array spectrophotometer.

NO• Scavenging Assay by HbO_2 Using Anaerobic Sequential Stopped Flow and Preincubation of the NO• with PEG-HCCs. NO• radicals were incubated with the PEG-HCCs at three different aging times before mixing with HbO_2 using the sequential mode of an Applied Photophysics model SX-18MV stopped-flow instrument with a rapid-scan diode array accessory. The instrument was located inside an anaerobic chamber model 110 V equipped with a gas analyzer model 10 (Coy Laboratory Products) for tracking the H_2 and O_2 levels. The chamber was filled with 10% H_2 in N_2 (4). The following stock solutions were prepared using TEA (50 mM, pH 7.4): PEG-HCCs (194.4 nM or 0.08 mg/mL) in aerobic buffer, NO• (20 μM) in anaerobic buffer and HbO_2 (15 μM) in air-saturated buffer. Either buffer (control) or PEG-HCCs were incubated with NO• in a 1:1 ratio for 20 ms, 1 s, or 1 min in the aging loop after first mixing. This solution was then further mixed with HbO_2 in a 1:1 ratio in the detector chamber. Note that 0.04 mg/mL (97.2 nM) was the concentration of the PEG-HCCs when it was incubated with NO• and 0.02 mg/mL (48.6 nM) when it was mixed with HbO_2, thus an overall 4× dilution after two-stage mixing.

$ONOO^-$ Scavenging Assay. A 12-mL aqueous solution containing 4 g $NaNO_2$ was added to a 125-mL aqueous solution containing 7.5 mL 35% H_2O_2 and 2 mL 96% H_2SO_4. This mixture was immediately poured into 250 mL solution containing 12.5 g NaOH. To remove unreacted H_2O_2, MnO_2 was added to the final mixture and allowed to stand for 15 min. The concentration of $ONOO^-$ was determined to be 1.73 mM using the absorbance maximum at 302 nm (ε = 1,670 $M^{-1}{\cdot}cm^{-1}$). $ONOO^-$ was portioned into 1.5-mL aliquots and frozen; during use, the $ONOO^-$ was kept on ice. Solutions of antioxidants (ascorbic acid, caffeic acid, PEG-HCCs, and Trolox) were prepared at 0.100 mg/mL (243.0 nM) in phosphate buffer. Pyrogallol red was prepared at 5 mM in phosphate buffer. Assays were done in methacrylate cuvettes. Typically, 25 μL of pyrogallol red stock solution was added to the cuvette, followed by phosphate buffer, antioxidant (10, 30, 50, 100, or 200 μL) and finally $ONOO^-$ (the final volume was 1.5 mL). The cuvette was inverted several times after the addition of the $ONOO^-$. The absorbance spectrum from 300–700 nm was measured. Each assay was carried out in triplicate. Control samples were the pyrogallol red in the absence of antioxidant and $ONOO^-$, as well as pyrogallol red in the presence of $ONOO^-$ without antioxidant. The absorbance change at 540 nm (ε = 2.4 × 10^4 $M^{-1}{\cdot}cm^{-1}$) was used in inhibition calculations (5).

1. Berlin JM, et al. (2010) Effective drug delivery, in vitro and in vivo, by carbon-based nanovectors noncovalently loaded with unmodified Paclitaxel. *ACS Nano* 4(8): 4621–4636.
2. Xia Y, Tsai A-L, Berka V, Zweier JL (1998) Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J Biol Chem* 273(40):25804–25808.
3. Petlicki J, van de Ven TGM (1998) The equilibrium between the oxidation of hydrogen peroxide by oxygen and the dismutation of peroxyl or superoxide radicals in aqueous solutions in contact with oxygen. *J Chem Soc, Faraday Trans* 94: 2763–2767.
4. Berka V, Wu G, Yeh H-C, Palmer G, Tsai A-L (2004) Three different oxygen-induced radical species in endothelial nitric-oxide synthase oxygenase domain under regulation by L-arginine and tetrahydrobiopterin. *J Biol Chem* 279(31):32243–32251.
5. Feelisch M, Noack EA (1987) Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylatecyclase. *Eur J Pharmacol* 139(1): 19–30.

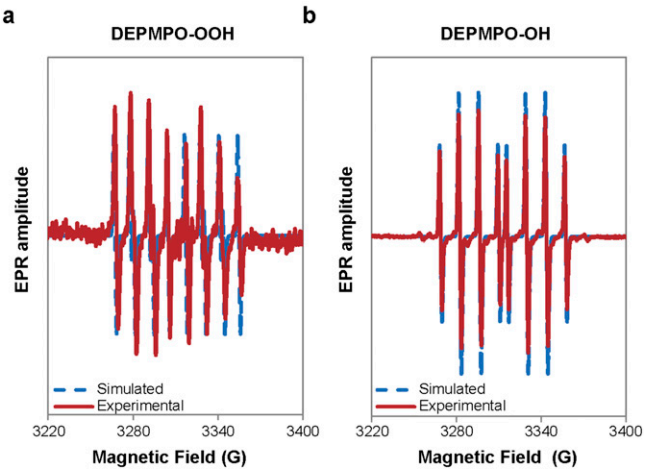

Fig. S1. Simulated and experimental spectra of the spin-adducts. (*A*) DEPMPO-OOH and (*B*) DEPMPO-OH adducts.

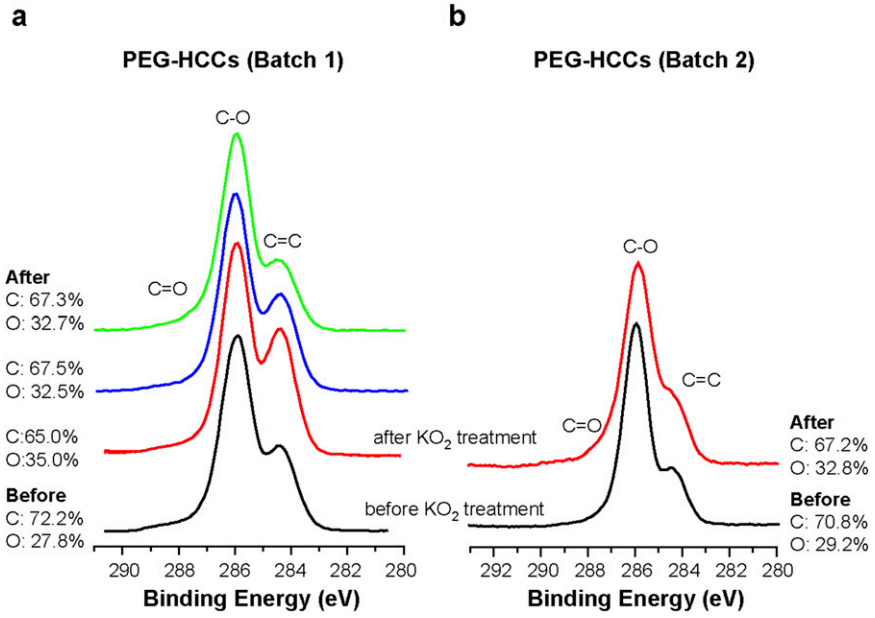

Fig. S2. XPS analysis of PEG-HCCs before and after treatment with KO_2. (*A*) PEG-HCC concentration = 0.350 mg/mL. (*B*) PEG-HCC concentration = 0.733 mg/mL.

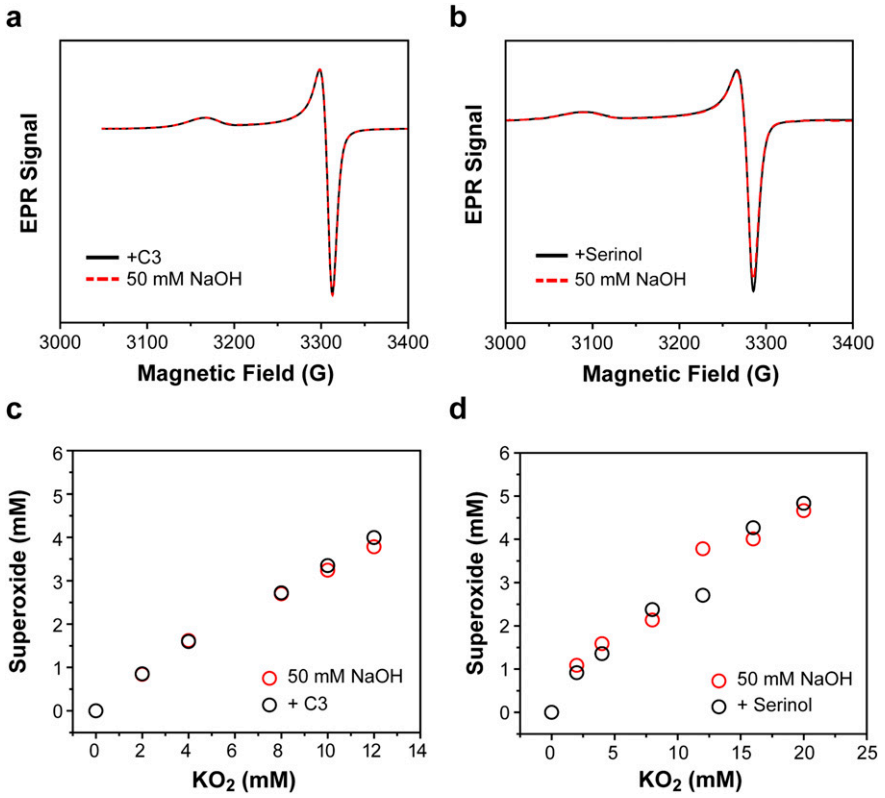

Fig. S3. Detection of $O_2^{\bullet-}$ from KO_2 in C_3 and C_{60}-serinol. Detection in the absence and presence of (*A* and *C*) C_3 or (*B* and *D*) C_{60}-serinol.

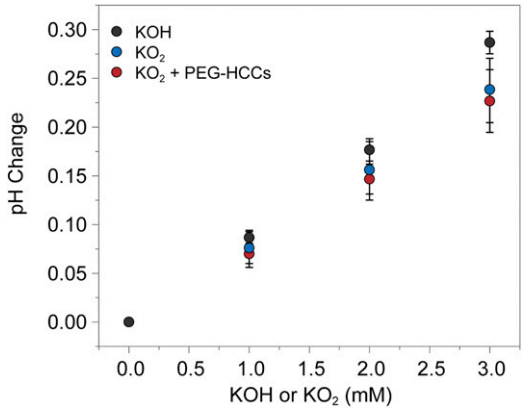

Fig. S4. Production of OH^- and its stoichiometry determined by pH shift. KOH or KO_2 (1, 2, or 3 mM) was added to 5 mL 20 mM Hepes at pH 7.2 (with or without 0.6 μM PEG-HCCs), and the pH increase due to OH^- production was measured. The error bars are the SDs of five measurements. The measured pH shift from KOH addition matches that calculated from the Henderson–Hasselbalch relationship from a strong base.

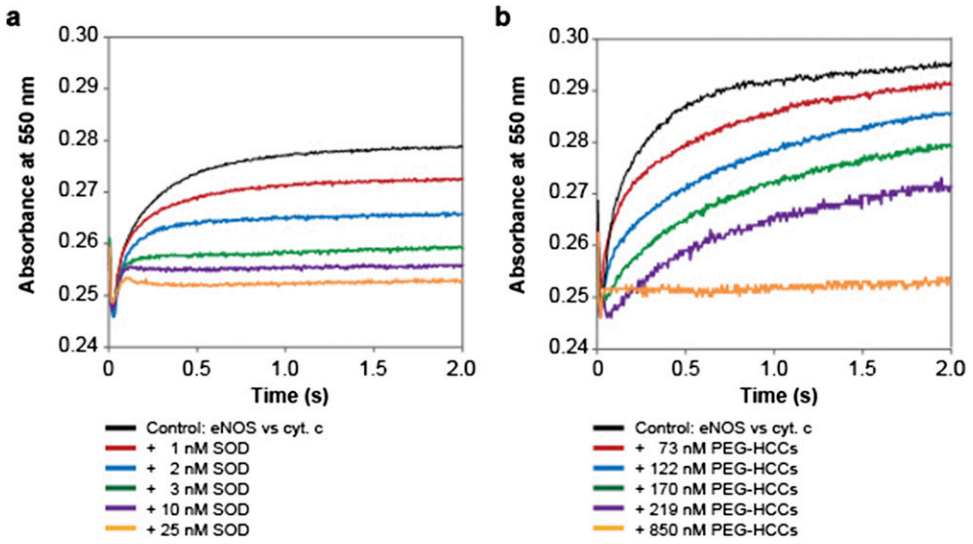

Fig. S5. Comparison of $O_2^{\bullet -}$ quenching activity of (*A*) SOD and (*B*) PEG-HCCs at pH 7.7 by competitive cytochrome *c* reduction. The concentration of $eNOS_{ox}$ and cytochrome *c* was 10 μM and 15 μM, respectively. The absorbance values of the reduced cytochrome *c* were converted to EC_{50} values and plotted in Fig. 7*A*.

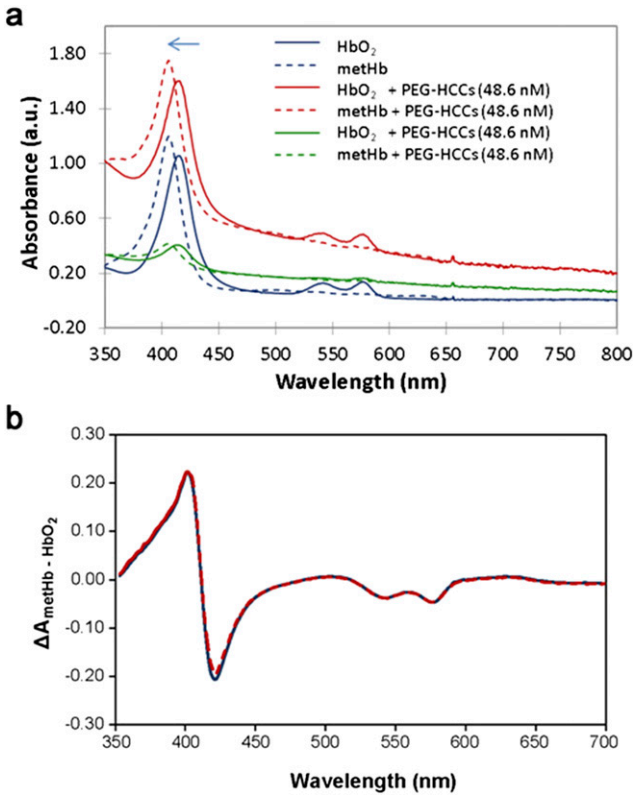

Fig. S6. Hemoglobin-based assays for $NO^{\bullet}$. (*A*) Oxyhemoglobin assay. UV-visible spectra of the (HbO_2) and final product (metHb) after manual addition of $NO^{\bullet}$ alone (blue set) or in the presence of two concentrations of PEG-HCCs (red and green sets) are shown. The arrow indicates the blue shift of the Soret peak corresponding to conversion of HbO_2 to metHb. Experiments were carried out in triplicate with experimental error ~10%. (*B*) Difference spectrum of metHb and HbO_2 (data from Fig. S5*A*) in the absence and presence of the PEG-HCCs (0.02 mg/mL or 48.6 nM). The difference between the $\Delta A_{401-411nm}$ values of the control and the $\Delta A_{401-411nm}$ values of PEG-HCCs treated system is lower than 5%. Control or PBS-treated system (blue, —), PEG-HCCs-treated system (red, —). The experiment was run in triplicate.

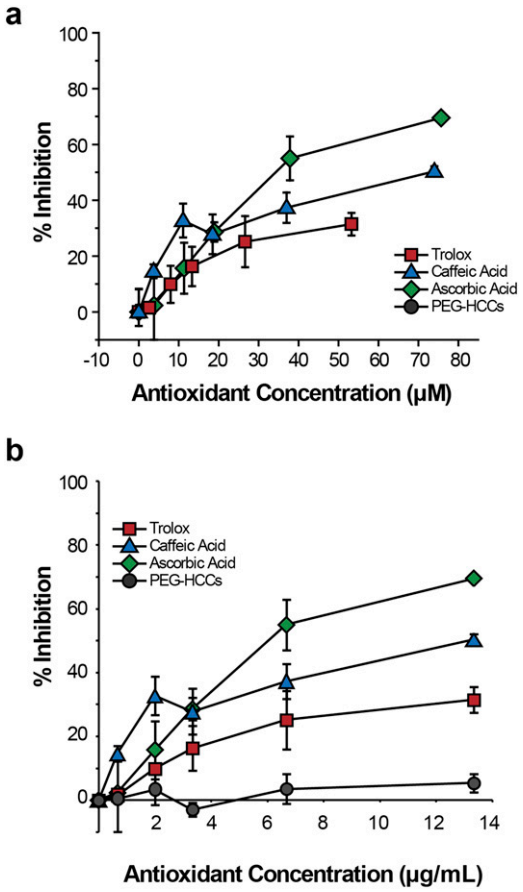

Fig. S7. The interaction between the $ONOO^-$ and PEG-HCCs. Inhibition of pyrogallol red decomposition: (◆) ascorbic acid, (▲) caffeic acid, (■) Trolox, and (●) PEG-HCCs. (*A*) Antioxidant concentration in microlar (note that the concentration of PEG-HCCs in this plot ranges from 0 to 0.034 μM and so is too small to be visible in *A*. (*B*) Antioxidant concentration in micrograms per milliliter. For experimental details see *SI Methods*, *$ONOO^-$ Scavenging Assay*.

According to the TGA plots

- Weight loss of the HCC: 58
- Weight loss of the PEG-HCCs: 84

$$\text{HCCs amount} = 100 - 84 = 16$$

$$\text{moles of HCCs} = \frac{16}{12\frac{g}{mol}} = 1.33 \text{ moles} \quad \text{(The O atoms were ignored, MW of carbon = 12 g/mol)}$$

$$\text{Functionality in HCCs} = \left(\frac{58}{100-58}\right) * (100-84) = 22.08$$

$$\text{Amount of PEG} = 84 - 22.08 = 61.92$$

$$\text{moles of PEG} = \frac{61.92}{5000\frac{g}{mol}} = 0.0124 \text{ moles}$$

$$\text{Ratio of functionalization} = \frac{\text{moles of C}}{\text{moles of PEG}} = \frac{1.33}{0.0124} \approx 107$$

1 PEG every 107 carbons

Molecular weight

Assumption: 137 carbons atoms/nm --------> 40 nm HCCs --------> 5480 C atoms

$$\#\text{of PEG chains} = \frac{5480}{107} = 51$$

$$\textbf{Molecular weight of PEG} - \textbf{HCCs} = \left(5480 * 12\frac{g}{mol}\right) * \frac{22.08}{16} + \left(5480 * 12\frac{g}{mol}\right) + \left(51 * 5000\frac{g}{mol}\right) = \mathbf{411509}\,\frac{\mathbf{g}}{\mathbf{mol}}$$

$$\textbf{Molecular weight of HCCs} = \underbrace{\left(5480 * 12\frac{g}{mol}\right)}_{\text{Carbon core}} + \underbrace{\left(5480 * 12\frac{g}{mol}\right) * \frac{22.08}{16}}_{\text{Functionality}} = \mathbf{156509}\,\frac{\mathbf{g}}{\mathbf{mol}}$$

Fig. S8. MW estimation of the PEG-HCCs.

Table S1. Hyperfine constants used in simulating the EPR spectra of the DEPMPO-OOH and DEPMPO-OH adducts

Adduct	Element	No.	Isotope	Spin	Abundance	a, G
	P	1	31	1/2	100.00	49.80
	N	1	14	1	99.64	13.30
DEPMPO-OOH			15	1/2	0.36	19.00
	H	1	1	1/2	99.99	11.00
g = 2.00504			2	1	0.01	1.40
Lorentzian/Gaussian:1.0 (meaning fully Lorentzian)	H(r)	1	1	1/2	99.99	1.00
linewidth: 1.2 G						
			2	1	0.01	0.14
	P	1	31	1/2	100.00	47.55
DEPMPO-OH	N	1	14	1	99.64	14.00
			15	1/2	0.36	21.00
g = 2.00455	H	1	1	1/2	99.99	13.20
Lorentzian/Gaussian:1.0 (meaning fully Lorentzian)			2	1	0.01	1.80
linewidth: 1.5 G						

Table S2. Relative percent of antioxidant activity

Sample		Average of the EPR amplitude[†]	SD	SE	Relative % of antioxidant activity
	Control	28,782	3,833	2,213	NA
$O_2^{\bullet -}$	PEG	23,774	6,408	3,700	13 ± 30[§]
	PEG-HCCs[‡]	433	320	185	98 ± 16[§]
	Control	15,242	3,594	2,075	NA
$^{\bullet}OH$	PEG[¶]	12,277	1,488	1,052	19 ± 5[§]
	PEG-HCCs	4,165	499	288	73 ± 20[§]

Calculated from [% = ((Control amplitude – PEG-HCCs or PEG amplitude)/Control amplitude) ×100%]. Experiments were carried out in triplicate. A quality control sample was run every three samples. NA, not applicable.
[†]EPR amplitude of the signal at 3,278 G and 3,308 G for the $O_2^{\bullet -}$ and $^{\bullet}OH$ scavenging experiments, respectively.
[‡]Absolute value.
[§]Error propagation from arithmetic calculations using SDs.
[¶]Experiment run in duplicate.