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Generation of Hydroxyl Radicals from Metal-Loaded Humic Acids

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Humic acids (HAs) are naturally occurring biopolymers that are ubiquitous in our environment. They are most commonly found in the soil, drinking water, and a variety of plants. Pharmacological and therapeutic studies involving humic acids have been reported to some extent. However, when certain transition metals are bound to humic acids, e.g., iron and copper, they can be harmful to biological organisms. For this study, humic acids were extracted from German, Irish, and New Hampshire soils that were selectively chosen because of their rich abundance in humic material. Each sample was treated at room temperature with 0.1 M ferric and cupric solutions for 48 h. The amount of iron and copper adsorbed by humic acid was accurately quantitated using atomic absorption spectroscopy. We further demonstrate that these metal-loaded humic acids can produce deleterious oxidizing species such as the hydroxyl radical (HO•) through the metal-driven Fenton reaction. Electron paramagnetic resonance (EPR) employing spin trapping techniques with 5,5-dimethylpyrroline *N*-oxide (DMPO) is used to confirm the generation of hydroxyl radicals. The DMPO−OH adduct with hyperfine splitting constants $A_N = A_H = 14.9$ G is observed upon the addition of exogenous hydrogen peroxide. The concentration of hydroxyl radical was determined using 4-hydroxytempo (TEMPO−OH) as a spin standard. The presence of another oxidizing species, $Fe{=}O^{2+}$, is also proposed in the absence of hydrogen peroxide.

Introduction

Humic substances (HS) are ubiquitous, heterogeneous biopolymers that are isolated from many types of terrestrial and aqueous environments. They are amorphous, organic materials that possess a variety of physical and chemical properties that make them unique to other types of environmental substances. The source of HS in the natural environment is not known; however, there are two principle models for the biosynthesis of HS. The first results from the autoxidation and condensation of lignin and polyphenols in plant matter in the humification process (*1*). The second model is the in vivo biosynthesis in plants, possibly during senescence. Recently, HS have been extracted from several living plants including tobacco, tomato, and peat (*2*).

Humic acids (HAs) are a subclass of HS. They are most abundant in the top 1−2 ft of the Earth's crust, where they interact with air and water. The functional groups of HAs are amine, carboxylic, carbonyl, phenol, catechol, and quinone (*3*−*5*). These groups are on the HA surface or are chemically combined to cross link the molecular backbone. To date, the structure for HA is unknown, only proposed building blocks exist (Figure 1). Although there are discrepancies among these proposed building blocks, similarities exist pertaining to the number and type of organic functional groups in each of the structures.

The environmental and physiological impact on humans and animals has been studied to some degree. HAs are known to exist in the gastrointestinal tract of humans and animals (*6*). They circulate with the blood (*7*) and are metabolized by the liver (*8*). Oral doses of HA reduce heavy metal adsorption in animals (*7*) and also decrease pesticide toxicity (*9*). There are also reports that link HA with cancer (*10*). Chlorinated HAs where the carbon-to-chlorine ratio is equal to 1 (C:Cl = 1) have been shown to be potent carcinogens in laboratory animals inducing a variety of liver and kidney tumors (*11*). These HAs are common to drinking water; however, isolated HAs are always partially chlorinated during the extraction process and may display some level of tumorgenesis (*12*−*17*). Respiratory hazards of HAs have also been studied. HAs have been isolated by alkali extraction of cigarette smoke condensate (*18*). It is believed that HAs reside in the lungs of cigarette smokers due to the incomplete combustion of tobacco (*18*).

HAs have a strong affinity toward metal cations and therefore can serve as effective chelators for numerous transition metal ions, in particular iron and copper. It has been suggested that the deposition of carbonaceous particulates, possibly HA, in the respiratory tract correlates with emphysema and pneumoconiosis as well iron and copper accumulation (*19*) and free radical generation (*20*). A possible mechanism for these deleterious effects is the generation of hydroxyl radicals (HO•) through the Fenton or the metal-catalyzed Haber−Weiss reaction and subsequent oxidation reactions involving hydroxyl radicals:

$$\mathrm{Fe(II)} + \mathrm{O_2} \rightarrow \mathrm{Fe(III)} + \mathrm{O_2^{\bullet -}} \quad (1)$$

$$\mathrm{HO_2^{\bullet}} + \mathrm{O_2^{\bullet -}} + \mathrm{H^+} \rightarrow \mathrm{O_2} + \mathrm{H_2O_2} \quad (2)$$

$$\mathrm{Fe(II)} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe(III)} + \mathrm{OH^-} + \mathrm{HO^{\bullet}}\ \text{(Fenton)} \quad (3)$$

The hydroxyl radical is a very potent and toxic oxidizing species. When they are produced in biological systems, hydroxyl radicals will react with biomolecules, proteins, and lipids at diffusion-controlled rate constants (*21*). Hydroxyl radicals have the ability to hydroxylate DNA and RNA, thus affecting cell processes such as base pairing, transcription, and translation (*22*).

The hydroxyl radical is a short-lived free radical that can be detected by using EPR spin-trapping techniques with nitroso and nitrone compounds. The most commonly used spin trap is 5,5-dimethylpyrroline *N*-oxide (DMPO), which yields stable nitroxyl radicals that are EPR active at room temperature. In this paper, we focus on the production, detection, and quantitation of hydroxyl radicals in several types of iron- and copper-loaded HAs. Until now, there has been no direct EPR evidence of hydroxyl radicals from humic-like materials. We will show that the production of the hydroxyl radicals is confirmed by radical scavengers such as dimethyl sulfoxide (DMSO) and inhibited by the enzymatic activity of catalase.

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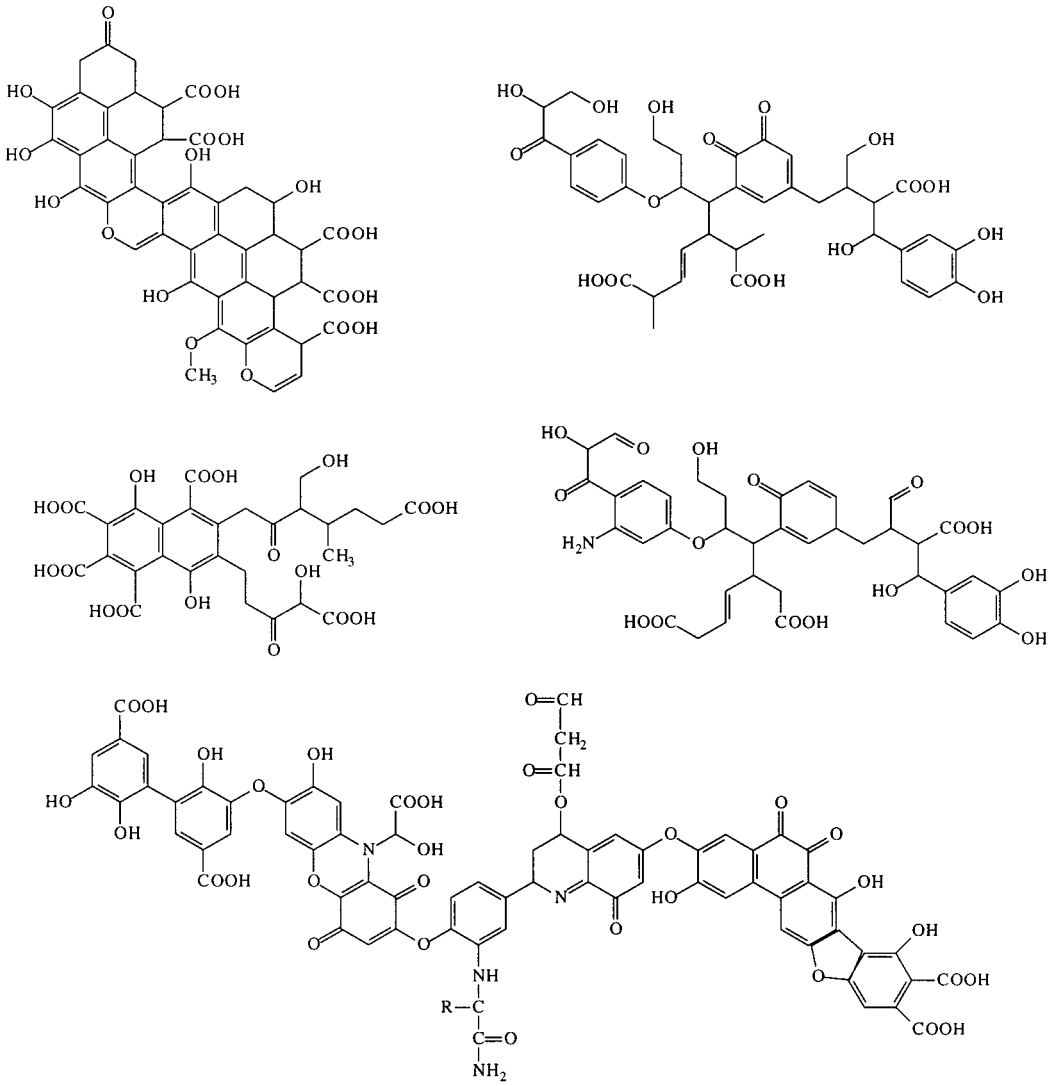

FIGURE 1. Proposed building blocks of HA.

Experimental Section

Reagents and Materials. Ferric ammonium sulfate, $Fe(NH_4)(SO_4)_2 \cdot 6H_2O$, and cupric sulfate, $CuSO_4 \cdot 5H_2O$, were purchased from Fisher Scientific (Pittsburgh, PA) and were ACS reagent grade. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and purified further by filtration with activated carbon. Hydrogen peroxide (30 wt %/wt), dimethyl sulfoxide, and sodium phosphate were also purchased from Aldrich Chemical Co. Catalase and superoxide dismutase (SOD) from bovine liver were purchased from Sigma Chemical Company (St. Louis, MO). 4-Hydroxytempo was also purchased from Sigma and was used as a spin-standard. The Davies group at Northeastern University provided humic acid samples from different sources. All solutions that were prepared for the spin-trapping experiment had a total volume of 1.0 mL and contained 7 mg of metal-loaded humic acid, 100 mM DMPO, 1 mM H_2O_2, and 5% v/v DMSO (when added) in 100 mM phosphate buffer, pH 7.4. The phosphate buffer was continuously agitated with Chelex beads for several hours until the time of the experiment. This was done to remove any trace metal contaminants that are commonly found in phosphate salts. All solutions, except the phosphate buffer, were prepared immediately prior to the start of the experiment.

Experimental Instrumentation. *Ultraviolet−Visible Spectroscopy.* A Hewlett-Packard 8453 UV−Vis spectrometer with a diode array detector was used to determine the concentrations of the stock solutions of DMPO and hydrogen peroxide. The concentration of DMPO was determined from its absorbance at λ_{max} of 234 nm with $\epsilon_{234} = 7700\ M^{-1}\ cm^{-1}$. The hydrogen peroxide solution was prepared by diluting the 30% (wt/wt) stock solution of which the concentration was measured by using the molar absorptivty at 240 nm with an $\epsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$. Hewlett-Packard spectrometer cells were UV-grade quartz with a 10-mm path length and a volume of 3 mL.

Electron Paramagnetic Resonance. EPR spectra were recorded at 295 K with a Bruker (Billerica, MA) EMX spectrometer operating at 9.7 GHz using a quartz aqueous flat cell with a total volume of 150 μL in a TM110 cavity. A modulation frequency of 100 kHz was used, with a scan range of 100 G and a scan time of 163.63 s. A microwave power of 20 mW and a modulation amplitude of 1.0 G were employed for the experiments. All experiments were run in triplicate.

Metal-Loading Protocol. Each metal salt was dissolved in doubly distilled, deionized water, and the final pH was adjusted to pH 2.0 with HNO_3 to avoid cation hydrolysis and polymerization, especially for iron(III). Each 0.100 M stock solution was stirred overnight at room temperature to ensure hydrolytic equilibrium. Each solution contained 100 mg of HA/100 mL of 0.100 M metal stock solution and was stirred for 48 h at 25 °C. Each solid product was separated by centrifugation, washed five times for 5.0 min with DI water, and freeze-dried.

TABLE 1. Metal Content of Humic Acid

humic acid	origin	wt %/wt (± 0.02)
Fe−NHA	New Hampshire soil	1.60
Fe−GHA	German soil	1.20
Fe−IHA	Irish peat	1.20
Cu−NHA	New Hampshire soil	1.10
Cu−GHA	German soil	1.00
Cu−IHA	Irish peat	0.95

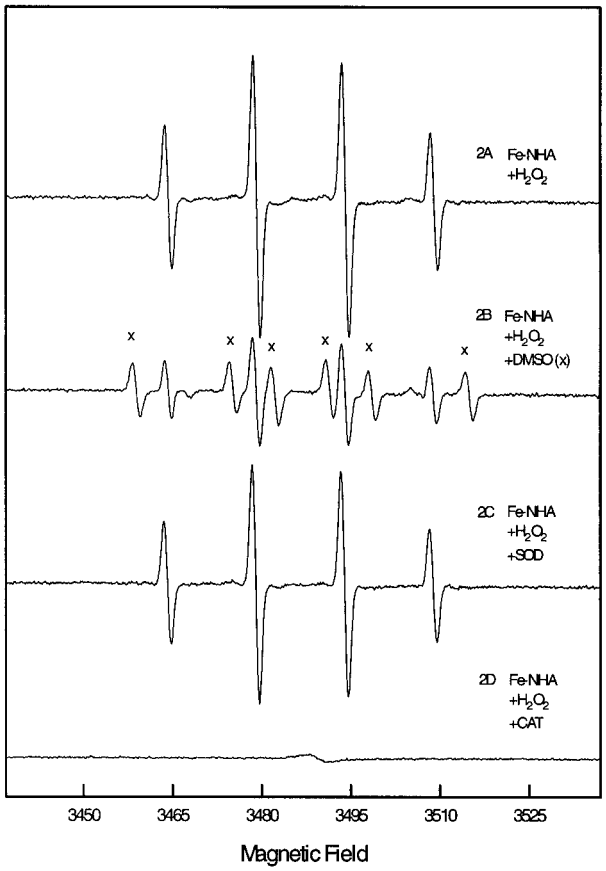

FIGURE 2. EPR Spectra of Fe−NHA in 100 mM phosphate buffer, pH 7.4, and 100 mM DMPO in the presence of (A) 1 mM H_2O_2, (B) As in panel A with 5% (v/v) DMSO. The (x) indicates the DMPO−CH_3 radical. (C) As in panel A with excess SOD. (D) As in panel A with an excess of catalase. These findings are typical for the other iron- and copper-loaded humic acids. All experiments were run in triplicate.

Atomic Absorption Spectrometry. Atomic absorption (AA) spectroscopy was used to quantitate the iron and copper species present in the several different metal-loaded HAs. For these studies, a Buck Scientific (East Norwalk, CT) 200A atomic absorption spectrometer was used. Iron and copper were analyzed at 324.7 and 248.3 nm, respectively. An air acetylene oxidizing flame was used for the analysis of all the samples.

Results and Discussion

Three types of HA samples were used for the course of this study. The extraction procedure and the specificity of the origin for each sample has been published elsewhere and therefore will not be discussed here (*23*). Atomic absorption spectroscopy was utilized to determine the total percentage (wt/wt) of the iron- and copper-loaded HA samples. The iron and copper content of the metal-loaded HA samples used for the spin-trapping experiment ranged from 1.20% to 1.60% for iron and from 0.95% to 1.10% for copper. These values are also reported in Table 1.

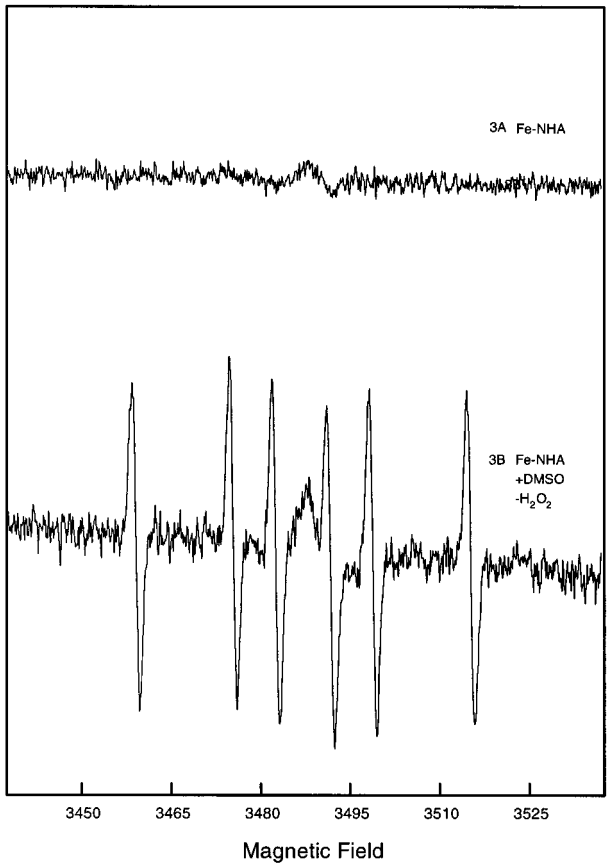

FIGURE 3. EPR spectra of Fe−NHA in 100 mM phosphate buffer, pH 7.4, and 100 mM DMPO: (A) no addition, (B) 5% (v/v) DMSO.

The reaction mixture that consisted of 100 mM DMPO, 1 mM H_2O_2, and 7 mg of ferric-loaded HA in phosphate buffer, pH 7.4, produced an EPR-active adduct that was characteristic of DMPO−OH with hyperfine lines $A_N = 14.9$ G and $A_H = 14.9$ G (Figure 2A). However, the nucleophillic addition of water on DMPO is known to rapidly occur in the presence of metal salts, particularly ferric salts, which results in an artifact that has the same spectral characteristics as the trapped hydroxyl radical. In light of this, there was concern that the DMPO−OH signal we observed was artificial. The metal loading HA protocol exclusively used ferric iron from ferric ammonium sulfate, and it is well-known that the production of hydroxyl radicals in Fenton chemistry requires ferrous iron. However, HAs are rich in catechol- and quinone-like functional groups, which are well-known to have oxidation and reduction properties. It is known that upon metal loading there is a reduction of ferric to ferrous ions near or in some of these sites. Mössbauer studies confirm that as much as 5% of the total weight of iron bound to HA during the metal-loading process using Fe^{3+} is reduced to Fe^{2+} (*24*). Thus, it is apparent HAs can create their own active-reducing Fe^{2+} species. Nevertheless, the radical scavenger DMSO was incorporated into the reaction mixture to verify that the production of hydroxyl radicals is genuine. The addition of DMSO to the DMPO, iron-loaded HA, and H_2O_2 solution produced additional hyperfine lines with $A_N = 16.3$ G and $A_H = 23.3$ G (Figure 2B). These spectral features are characteristic of the DMPO−CH_3 radical. Methyl radicals ($^{\bullet}CH_3$) are produced and then trapped by DMPO when the hydroxyl radical reacts with DMSO. The addition of SOD did not cause an observable increase in the DMPO−OH signal for either the iron- or copper-loaded HA samples, thus

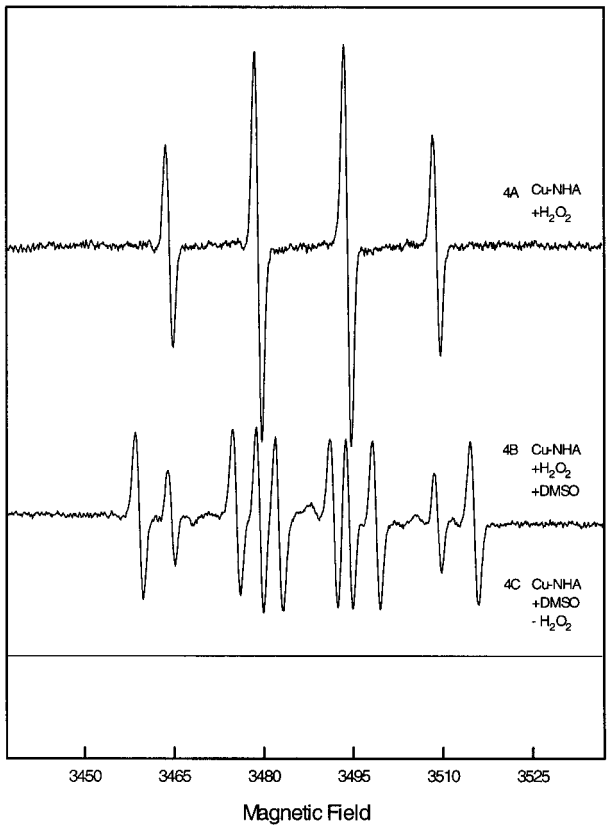

FIGURE 4. Spectral subtraction of Cu−NHA from control. All solutions contained 100 mM DMPO in phosphate buffer, pH 7.4, and (A) 1 mM H_2O_2, (B) as in panel A with 5% (v/v) DMSO, (C) 5% (v/v) DMSO in the absence of H_2O_2.

indicating that superoxide ($O_2^{\bullet-}$) does not partake in the reaction or in the formation of H_2O_2 (Figure 2C). The DMPO−OH signal was suppressed when catalase was added to the reaction (Figure 2D), indicating that the primary radical present was the hydroxyl radical.

As expected, the solution mixture that contained 100 mM DMPO and 7 mg of the iron-loaded HA without the addition of 1 mM H_2O_2 did not give an EPR-active species (Figure 3A). However, the reaction mixture that consisted of 100 mM DMPO, 7 mg of iron-loaded HA, and 5% (v/v) DMSO in the absence of H_2O_2 produced a signal that was indicative of trapped methyl radicals by DMPO (Figure 3B). The hyperfine coupling constants and spectra overlap suggest that the carbon-centered radical trapped by DMPO when DMSO was added in the presence/absence of H_2O_2 are one and the same. This indicates the presence of another oxidizing species in addition to the hydroxyl radical that is capable of oxidizing the relatively unreactive DMSO. There are reports which suggest that the autoxidation of ferrous ions occur in phosphate buffer systems, especially under slightly alkaline conditions (*25*, *26*). The oxo-iron species that may form are the ferryl and/or perferryl species. These species have the ability to oxidize DMSO, resulting in the EPR-active DMPO−CH_3 adduct that is observed when H_2O_2 is not present in the reaction. The DMPO−CH_3 signal is reduced by almost 50% (data not shown) when the reaction mixtures were purged with argon for 15 min. This indicates that molecular oxygen may partake, but is not essential, in the formation of the oxidizing species. The role of molecular oxygen may be similar to that found in the enzymatic pathway speculated for cytochrome P450. The radical species formed and trapped by DMPO under anoxia suggests the possibility of an electron transfer from the HA followed by a methyl radical displacement.

TABLE 2. Signal Intensity of DMPO−OH and Relative Concentration of $HO^{\bullet}$

sample	signal intensity	relative [$HO^{\bullet}$] using spin-standard 4-hydroxytempo (±0.03)
Fe−NHA	3.88×10^4	2.36 μM
Fe−IHA	2.23×10^4	1.34 μM
Fe−GHA	2.24×10^4	1.31 μM
Cu−NHA	2.83×10^4	1.72 μM
Cu−IHA	2.45×10^4	1.58 μM
Cu−GHA	2.21×10^4	1.48 μM

The same studies were also performed with copper-loaded HA samples. Again, there was an indication that hydroxyl radicals were produced when H_2O_2 was added to the reaction mixture that contained DMPO and copper-loaded HA (Figure 4A). However, the control that consisted of DMPO and the copper-loaded HAs had an EPR spectra that was characteristic of DMPO−OH with hyperfine lines $A_N = 14.9$ G and $A_H = 14.8$ G. This species is most likely to result from the nucleophilic addition of H_2O in the presence of surface HA cuprous ions. Spectral subtraction of the Cu-loaded HAs from their corresponding controls was utilized to determine the relative amount of hydroxyl radicals trapped by DMPO. Dimethyl sulfoxide was used to confirm the authenticity of the hydroxyl radicals being trapped (Figure 4B). Unlike the iron-loaded HAs, a trapped methyl radical was not observed in the absence of H_2O_2 for the copper-loaded HAs (Figure 4C).

The intensity of the DMPO−OH signal is comparative for all of the iron- and copper-loaded HAs used for this study. The spin-standard, 4-hydroxytempo, was used to determine the relative concentrations of hydroxyl radicals produced and trapped by DMPO from the metal-loaded HAs (Table 2).

The analysis of the [$HO^{\bullet}$] vs metal content of the various metal-loaded HAs indicate that copper-loaded HAs are more reactive than iron-loaded HAs/g. Generally, copper is present in lower concentrations than iron in biological organisms. However, copper ions chelated to HAs may be more toxic to biological systems than iron-loaded HAs.

These studies indicate that the deposition of HA particulates in the respiratory tract, circulatory system, and tissue may have serious biological implications. Humic acids are naturally occurring throughout the environment, and biological organisms are exposed to these polymers through simple media such as the drinking water, plants, and also dust particulates in the air. HAs have a strong affinity for the transition metals iron and copper. When these metals are bound to HA, they can efficiently generate reactive oxygen species such as the hydroxyl radical, which is known to cause oxidative disease in biological systems.

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