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Evaluation of Hydroxyl Radical-Scavenging Abilities of Silica Hydride, an Antioxidant Compound, by a Fe^{2+}-EDTA-Induced 2-Hydroxyterephthalate Fluorometric Analysis

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ABSTRACT The hydroxyl radical scavenging capacity and efficacy of a novel organosiliceous anionic hydride compound, silica hydride, were quantified by a recently developed method. The method measures a direct relationship between the hydroxyl radical scavenging capability of the antioxidant compound and the linear decrease in signal from a fluorescent 2-hydroxyterephthalate product created by reacting an Fe^{2+}-EDTA complex in the presence of a potential radical scavenger. A fluorescence signal half-inhibition, IC_{50}, value of 1.4 ± 0.1 μM was obtained for silica hydride compounds. The validity of the analysis was verified by electron spin resonance spectroscopy, spectrophotometric analysis of NAD^+/NADH ratios, mitochondrial membrane potential measurements, and assays of both cytochrome $c(Fe^{3+})$ to cytochrome $c(Fe^{2+})$ and epinephrine to adenochrome reductions.

KEY WORDS: • *silica hydride* • *radical* • *antioxidant* • *microcluster* • *fluorescence*

INTRODUCTION

FREE RADICALS AND THE RESULTANT OXIDATIVE STRESS on an organism have been the subject of analysis for clinicians, nutritionists, biologists, and chemists alike. Of all the reactive oxygen species (ROS), the hydroxyl ($^{\cdot}OH$) ROS is known to be one of the most reactive and physiologically harmful,[1] suspected in such pathologies as atherosclerosis, oncogenesis, cataractogenesis, and DNA mutation.[2–4]

The investigation into the involvement of free radicals in the forementioned pathologies has been ongoing for decades. It has been demonstrated that the buildup of oxidized species occurs after the concentration of reaction products supercedes that at which the cell's natural antioxidant system can effectively neutralize the radical or oxidized product.[5] The excess of oxidized products in the body has been directly linked to numerous pathologies and represents a serious health issue.

The use of dietary antioxidant supplementation has been established as a potential protocol to decrease the detrimental effects of oxidative stress,[6] with some compounds being more effective than others. Yet most commercially marketed antioxidant compounds have not been characterized with their *in vitro* and *in vivo* ability to neutralize free radical species, and there is no FDA regulation on their use and distribution.

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The ability to effectively and accurately determine antioxidant capacity for hydroxyl free radicals has been a combative problem with scientists for some time. Even though there are a number of analytical techniques and indicators available, such as pH, oxidation reduction potential (ORP), electron spin resonance (ESR), chemimodification, and photosensitization, they maintain inherent limitations and practicalities.

The direct use of pH and reduction potential measurements (ORP) gives an indication of the probability of a compound to act as an antioxidant, but does not define any specificity toward any particular ROS nor does it address the induction of cytotoxicity.[7] For example, by strictly using direct reduction potential measurements, $LiAlH_4$ would appear to be a great antioxidant with its large capacity to decrease ORP, yet it reacts violently with aqueous solutions and is a toxic reducing agent.

Even though ESR spectroscopy is well established to measure the disappearance of ROS in a reaction, the methods used to analyze the radical species are not carried out under optimum biological conditions.[8] The Fenton reaction is commonly used to address the question of quantifying hydroxyl radical quenching efficacy, however, it is carried out at a pH that may hinder or bias the results.[9]

Photosensitizers, such as malachite green, effectively introduce ROS into cells for *in vitro* analysis. However, they have an inherent nonspecificity to introduce both hydroxyl and superoxide radical ($O_2^{\cdot -}$) species.[10]

To address these problems when studying hydroxyl radicals and antioxidant capacity, a new method was developed by Yang and Guo at Xiamen University utilizing the Fe^{2+}-

EDTA hydroxylation of terephthalate to directly measure the radical-scavenging ability of antioxidant compounds at biological pH without the addition of hydrogen peroxide (H_2O_2).[11]

This established a measurement of the decrease in fluorescence in direct relationship to hydroxyl radical scavenging capability by the following reaction scheme (Eqs. 1–4):

$$Fe^{2+}\text{-EDTA} + O_2 \longrightarrow Fe^{3+}\text{-EDTA} + O_2^{\cdot -} \quad (1)$$

$$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (2)$$

$$Fe^{2+}\text{-EDTA} + O_2^{\cdot -} + 2H^+ \longrightarrow Fe^{3+}\text{-EDTA} + H_2O_2 \quad (3)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \xrightarrow{k_0} Fe^{3+}\text{-EDTA} + OH^- + {}^{\cdot}OH \quad (4)$$

The premise for the assay follows the measurement of the fluorescent 2-hydroxyterephthalate (HOTP) created by the reaction of a Fe^{2+}-EDTA complex with molecular oxygen to produce a superoxide radical species that dismutates to H_2O_2 and then decomposes to form the hydroxyl radical. It is theorized that the greater the antioxidant capacity of the compound, the less hydroxyl radical present and the resultant less fluorescent adduct HOTP is produced, as depicted in Fig. 1. The stoichiometry of the reactions leads to a linear and direct relationship between the amount of antioxidant substrate and the measurement of the radical scavenging capacity by the following reactions (Eqs. 5 and 6):

$$\text{terephthalate} + {}^{\cdot}OH \xrightarrow{k_1} \text{HOTP} \quad (5)$$

$$\text{scavenger} + {}^{\cdot}OH \xrightarrow{k_2} \text{other products} \quad (6)$$

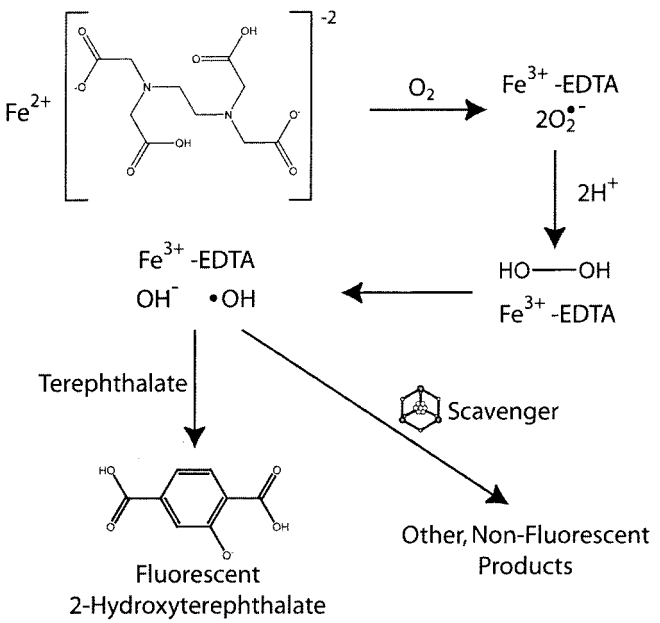

FIG. 1. Mechanism for the hydroxyl radical analysis. Fe^{2+}-EDTA is oxidized to produce Fe^{3+}-EDTA and superoxide, which is further reacted under acid catalysis to produce peroxide, which decomposes to a hydroxyl radical and a hydroxide ion. The hydroxyl radical reacts with terephthalate to produce the fluorescent adduct, 2-hydroxyterephthalate, emitting at 432 nm. Any scavenger present reacts with the hydroxyl radical creating other, nonfluorescent products. These interactions directly relate the concentration of the hydroxyl radical present with the fluorescent intensity.

This methodology was used to analyze a novel dietary antioxidant supplement, silica hydride.[12] The compound has demonstrated unique biochemical properties *in vitro*, including neutralizing ROS, acting as a catalyst for redox-coupled compounds, and decreasing lactic acid buildup after exercise *in vivo*.[13–16] The hydride compound is a biologically friendly organosiliceous silsesquioxane "cage" that acts as an electron source through interstitially imbedded hydrogen anions, or H^- ions, colloidally carrying the H^- through the body. Fig. 2 illustrates a conceptual model and transmission electron microscopic image of the silicate mineral hydride.

An interesting attribute of this compound is that although it is hydride anion-based, it does not react violently with water or any other tested solution, while maintaining a reduction potential of up to −850 mV[15] for extended lengths of time. Recent publications on silica hydride have shown its significant abilities to act as an antioxidant and internal energy producer, as well as clinically affecting cardiovascular responses to exercise by reducing lactic acid production and buildup.[13–16] Cytotoxicity testing of the compound concludes no toxic cellular effects or instigation of apoptosis or necrosis.

This work utilizes the recently developed Fe^{2+}-EDTA fluorometric analysis to quantify and compare the antioxidant properties and capabilities of silica hydride.

MATERIALS AND METHODS

Apparatus

The fluorometric analysis and relative fluorescence intensity were measured with an FP-750 spectrofluorimeter (Jasco, Inc., Easton, MD, U.S.A.) in a 1-cm quartz cuvette. Readings were acquired with 326 nm excitation and 432 nm emission wavelengths. The parameters of the spectrofluorimeter were set to ±5 nm bandpass for both the excitation and the emission wavelengths. The process was performed in triplicate.

Reagents

All reagents, unless otherwise noted, were from Sigma–Aldrich (St. Louis, MO, U.S.A.) and of analytical purity. A stock solution of 1.0 m*M* terephthalate was prepared by the addition of 16.7 mg of terephthalic acid in 100 mL of 10 m*M* NaOH solution. A 1.0 m*M* ferrous ion solution was prepared by the addition of ammonium ferrous sulfate in 5.0 m*M* H_2SO_4. Catalase, 1,090 U mg^{-1}, was prepared by a 1:10 dilution of stock catalase (10,900 U mg^{-1}) in 50 m*M* phosphate buffer solution at pH 7.4. A solution of 1.0 m*M* EDTA was also prepared in ddH_2O.

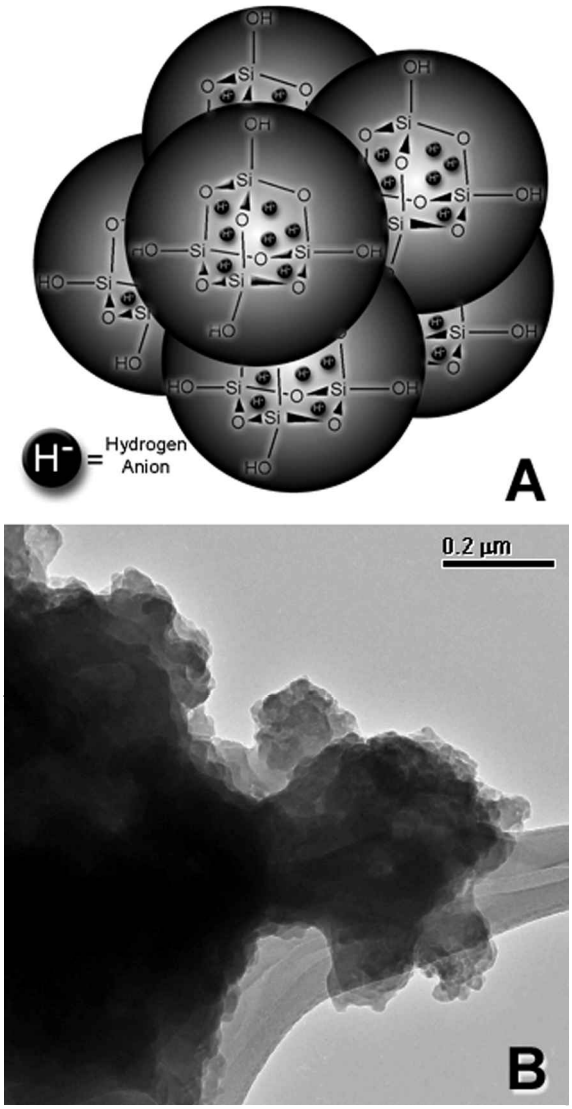

FIG. 2. Silica hydride. (**A**) Graphical model of silica hydride as small, submicron silsesquioxane spheres interstitially embedded with hydride anions. (**B**) A scanning transmission electron microscopic image of the silica hydride, taken with a Phillips CM30 instrument illustrating the small, spherical constituents of the silica hydride compound.

An analytical working solution of was prepared from the preceding stock solutions in a 10-mL graduated cylinder containing, as added in the following order: 1.0 mL of 0.1 m*M* terephthalate, 0.30 mL of 1.0 m*M* EDTA, 1.0 mL of [SCAVENGER], 0.30 mL of 1 m*M* Fe^{2+}, and 2.0 mL of 0.50 m*M* phosphate/catalase solution.

A working solution, [SCAVENGER], of silica hydride was prepared as previously described[12] and added to ddH_2O 15 min before its addition to the Fe^{2+}-EDTA analytical solution in the following incremental concentrations (μg/mL): 2.5, 5.0, 10.0, 25.0, 50.0, 75.0, 100.0, 125.0, 150.0, 175.0, 200.0, 225.0, 250.0, 275.0, and 300.0.

Upon the addition of the Fe^{2+} to the analytical solution, the mixture was allowed to sit at room temperature for 6 min, at which time the relative fluorescence intensities were measured.

Controls to determine the maximum and minimum fluorescence signal were obtained by the direct measurement of the analytical working solution without the scavenger. Additional spectra were obtained of the scavenger in the analytical working solution without the Fe^{2+} solution to check that no fluorescence was being emitted by the scavenger in the analytical solution.

RESULTS

The reaction scheme for the direct measurement of the hydroxyl radical scavenging capacity (Eqs. 7 and 8) is based on the principle that any molecule introduced into the analytical solution that is able to react with the ˙OH will compete with the terephthalate for ˙OH and therefore will reduce the concentration of the fluorescent HOTP produced in the reaction, decreasing the fluorescence signal.

$$\frac{F_0}{F} = 1 + \frac{k_2[C_{scav}]}{k_1[TP]} \tag{7}$$

$$\log\left(\frac{F_0}{F} - 1\right) = \log[C_{scav}] + \log\frac{k_2}{k_1[TP]} \tag{8}$$

where F_0 is the maximum fluorescence signal, F is the measured fluorescence signal for the scavenger introduced into the analytical sample, $[C_{scav}]$ is the concentration of the scavenger, and [TP] is the terephthalate concentration. Kinetic constants, k_1 and k_2, refer to reaction rates for [TP] and $[C_{scav}]$, respectively. As the [TP] is held constant, the equation reduces to $\log(F_0/F - 1) = \log[C_{scav}]$.

Plotting the log–log graph of the relative fluorescence signal, $\log(F_0/F - 1)$, as a function of the concentration of scavenger, $\log[C_{scav}]$, results in a linear trend. Figure 3 displays the results obtained for the silica hydride.

The concentration of the silica hydride producing the half-inhibition of the maximum fluorescence intensity (IC_{50}) was calculated by the extrapolation of $\log(F_0/F - 1) = 0$. This is possible because when $F = {}^1/_2\ F_0$, $\log(F_0/F - 1) = 0$. The calculated IC_{50} value for the compound is 1.4 ± 0.1 μM, indicating the concentration required to reduce 50% of the ROS present.

DISCUSSION

The analysis of the novel silica hydride by this newly developed technique distinctly conveys its effectiveness as an antioxidant against ˙OH ROS at biological pH. The experimental IC_{50} values for the silica hydride of 1.4 ± 0.1 μM illustrate the efficacy and potential as a "biochemical fuel cell" with the intrinsic stored energy of the reduced ORP and low IC_{50} value. Compared with other antioxidant compounds, silica hydride fairs very well. For example, the IC_{50} values for melatonin range from 0.16 to 0.66 m*M*, and pinoline from 0.04 to 0.13 m*M*.[17] Other antioxidants, such as

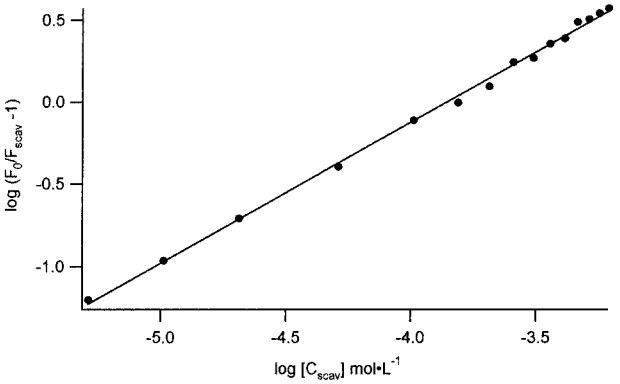

FIG. 3. Chart of the relative fluorescence signal, $\log(F_0/F - 1)$ as a function of the concentration of scavenger, $\log[C_{scav}]$, at 326 nm excitation and 432 nm emission wavelengths ±5 nm bandpass. Regression analysis of the averaged data for the three replicates ($\sigma = 1.5 \times 10^{-3}$) resulted in a linear trend [$f(x) = 0.857x + 3.303$] for the silica hydride, (●), correlation coefficient, $R^2 = 0.9979$. The concentration of silica hydride producing the half-inhibition of the maximum fluorescence intensity (IC_{50}) was calculated by the extrapolation of $\log(F_0/F - 1) = 0$. The IC_{50} value obtained is 1.4 ± 0.1 μM.

rhein ($IC_{50} = 64\ \mu M$), aloe-emodin ($IC_{50} = 65\ \mu M$), anthrone ($IC_{50} = 62\ \mu M$), dithranol ($IC_{50} = 72\ \mu M$), and rhein anthrone ($IC_{50} = 76\ \mu M$),[18] although indicating significant IC_{50} values, are less effective in this type of ROS assay. Different soybean isoflavone compounds, such as genistein with an IC_{50} value of 40 μM, were found to reduce oncogenesis and tumor growth in the human prostatic cancer cell line, LNCaP, presumably through the inhibition of oxidative stress.[19] The half-inhibition rate of the silica hydride compound comparatively indicates a promising use as a method for decreasing oxidative stress and potentially the resultant pathogenesis.

To validate the results obtained by this time-efficient, easy, and cost-effective method, additional tests were performed by outside sources to confirm the in-house, HOTP-fluorometric results. Three "traditional" analyses were performed by three major universities. Spectrophotometric analyses[20] indicate an increase in the NADH/NAD^+ ratio, in addition to an increase in mitochondrial membrane potential ($\Delta\Psi$). The cumulative results indicate the neutralizations of radical species by an electron donation from the hydride ion in solution within the siliceous silsesquioxane cage of the silica hydride.

The compound was shown to specifically reduce cytochrome *c* (Eq. 9) and NAD^+ (Eq. 10) by the following mechanisms[21]:

$$\text{Silica}(H^-) + \text{cyt } c(Fe^{3+}) \longrightarrow \text{Silica} + \text{cyt } c(Fe^{2+}) + H^+ \quad (9)$$

$$\text{Silica}(H^-) + NAD^+ \longrightarrow \text{Silica} + \text{NADH} \quad (10)$$

Additionally, silica hydride has been shown to inhibit the reduction of cytochrome *c* by superoxide (Eq. 11):

$$O_2^{\cdot -} + \text{cyt } c(Fe^{3+}) \longrightarrow O_2 + \text{cyt } c(Fe^{2+}) \quad (11)$$

indicating that the superoxide radical was reduced. Because silica hydride reduced the cytochrome *c* in (Eq. 6) and inhibited the cytochrome *c* reduction in (Eq. 8), an additional assay was performed to clear up any confusion about its role in radical reactions. The second assay observes the oxidation of epinephrine to adrenochrome by superoxide (Eq. 12):

$$O_2^{\cdot -} + \text{epinephrine} \longrightarrow H_2O_2 + \text{adrenochrome} \quad (12)$$

In Eq. 12, when the silica hydride was added, the superoxide was scavenged, leaving epinephrine, illustrating the antioxidant activity of the compounds.

Control assays performed using ESR were used to measure hydroxyl radical reduction.[22] The conclusion of the assay was that silica hydride demonstrates antioxidant activity toward hydroxyl radicals.

In light of the presentation of all of the data, it is thereby postulated that the mechanism of silica hydride against the hydroxyl radical (Eq. 13) is presumably:

$$\text{Silica}(H^-) + {}^{\cdot}OH \longrightarrow \text{Silica} + H_2O \quad (13)$$

where the mechanism follows the electron-rich, 1s1s′ orbital[23] of the hydride anion reacting with the electron-deficient ·OH radical as depicted mechanistically in Fig. 4.

These tests, in conjunction with the spectrofluorometric HOTP assay described in this article, concur with the results that the compound is indeed quenching the hydroxyl radical species reactions. The results of the reduction assays,

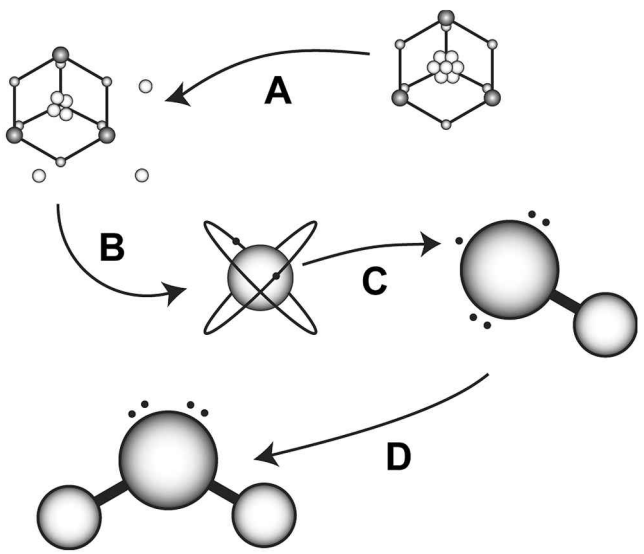

FIG. 4. Mechanism of hydroxyl radical with silica hydride. In solution, silica hydride slowly dissociates its embedded hydride anions (**A**), which interact with the 1s1s′ orbital of the hydride anion (**B**), directly reducing the unpaired electron of the hydroxyl radical (**C**), water, and $1s^1$ hydrogen. This mechanism results in no toxic products formed and does not induce a cytotoxic environment.

ESR, and the mitochondrial membrane potential assays of NADH are consistent with the results presented by the technique used in this article, validating its use as a method to quantify antioxidant activity. The resultant IC_{50} value for the silica hydride compound adds further support to its use as an antioxidant. As previously described by Yang and Guo,[11] this method provides an easy, effective, and cost-efficient assay for antioxidant capacity at biological pH.

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