Selective Detection of Hydroxyl Radical Scavenging Capacity Based on Electrogenerated Chemiluminescence Detection Using Tris(2,2′-bipyridine)ruthenium(III) by Flow Injection Analysis

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We describe a new method for detecting hydroxyl radical scavenging capacity based on tris(2,2′-bipyridine)ruthenium(III) [$Ru(bpy)_3^{3+}$] chemiluminescence and flow injection analysis. Hydroxyl radicals were generated by the Fenton reaction. The scavenging capacity of the six antioxidants tested decreased in the following order: edaravone>L-tryptophan>gallic acid>Trolox>*N*-acetyl-L-cysteine>ascorbic acid. The proposed method allowed a sample throughput of about 80 samples/h. The six antioxidants were found to inhibit chemiluminescence intensity of $Ru(bpy)_3^{2+}$. The proposed method is a rapid, selective, and accurate procedure for the study of hydroxyl radical scavenging capacity by $Ru(bpy)_3^{3+}$ chemiluminescence.

Key words hydroxyl radical; tris(2,2′-bipyridine)ruthenium(III); chemiluminescence; scavenging capacity; flow injection analysis

Oxygen-derived free radicals are thought to be involved in several diseases, such as cancer, cardiovascular diseases, and diabetes. Among various oxygen-derived free radicals, the hydroxyl radical (·OH) is one of the most highly reactive and harmful oxygen-derived free radicals in a living organism. When ·OH is generated in excess and the cellular antioxidant defense is deficient, some free radical chain reactions can attack proteins, lipids, and nucleic acids, leading to cellular damage.[1—3] Living systems contain complex enzymatic antioxidants such as superoxide dismutase, glutathione peroxidase, and catalase. These enzymes can block the initiation of ·OH and the free radical chain reaction.[4] There are also some important non-enzymatic antioxidants that can break free radical chain reactions.[5,6] Thus, in order to prevent several diseases, it is necessary to develop a simple and selective method for determining the ·OH scavenging capacity of various antioxidants.

Commonly used methods for detecting ·OH include electron spin resonance (ESR)[7—9]; chemiluminescence[10,11]; and high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection,[12] electrochemical detection (ED),[13—15] and fluorometric detection.[16,17] The ESR method has been widely accepted to measure ·OH scavenging capacity, but it requires expensive instrumentation and cannot be readily used to obtain quantitative estimates of ·OH adducts because the ·OH spin is unstable and may react with other products. The luminol chemiluminescence method has some advantages for ·OH determination, but the luminal used also reacts with superoxide and hydrogen peroxide, resulting in measurement errors. HPLC methods involve complicated procedures. Various reagents are used to trap ·OH to form a stable adduct. Then, the reagent and ·OH adducts are separated by HPLC, and the procedure is time consuming.

Chemiluminescence has become an important and valuable detection method in analytical chemistry in recent years because of its high sensitivity and wide linear dynamic range and its need for relatively simple instrumentation.[18] Tris(2,2′-bipyridine)ruthenium(III), or $Ru(bpy)_3^{3+}$, has been shown to be an important chemiluminescence reagent. When coupled with flow injection analysis (FIA),[19] chemiluminescence-based methods provide a simple, rapid, and reproducible means of detection.

Chemiluminescence is generated when $Ru(bpy)_3^{3+}$ comes in contact with hydroxide ion (HO^-).[20] It is suggested that ·OH generates excited $Ru(bpy)_3^{2+}$* in an electron transfer reaction with $Ru(bpy)_3^{3+}$. When this excited state decays to the ground state, the background emission is generated as follows:

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^-$$

$$Ru(bpy)_3^{3+} + \cdot OH \rightarrow [Ru(bpy)_3^{2+}]^* + 1/2\, O_2 + H^+$$

$$[Ru(bpy)_3^{2+}]^* \rightarrow Ru(bpy)_3^{2+} + h\nu$$

The ·OH radical is generated by the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$). $Ru(bpy)_3^{3+}$ and ·OH are in contact in the spiral cell in the detector, and thus, the chemiluminescence is continuously maintained. Constant chemiluminescence is generated and recorded as the background emission. The background emission decreases in proportion to the ·OH scavenging capacity. This reaction was used in the experiments described here.

In this study, we developed and applied a rapid, selective, and accurate procedure for the study of ·OH scavenging capacity by $Ru(bpy)_3^{3+}$ chemiluminescence.

Experimental

Reagents and Chemicals Tris(2,2′-bipyridine)ruthenium(II) chloride hexahydrate ($Ru(bpy)_3Cl_2 \cdot 6H_2O$) was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.) and was used without further purification. Iron(II) chloride tetrahydrate (99.9%), hydrogen peroxide (30%), sulfuric acid, and L-tryptophan were obtained from Wako Pure Chemical Industries (Osaka, Japan). Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), *N*-acetyl-L-cysteine, and ascorbic acid were obtained from Sigma-Aldrich Co. Pyrogallol-4-carboxylic acid (gallic acid) was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan). Distilled water was purified with Still Ace SA-2000E (Tokyo Rikakikai Co., Tokyo, Japan). Other chemicals used as reagents were of analytical or HPLC grade. A 3.0 mM H_2O_2 solution and 0.75 mM $FeCl_2$ solution were prepared daily with distilled water. Stock solutions (30 mM) of four samples (L-tryptophan, *N*-acetyl-L-cysteine, gallic acid, and ascorbic acid) were prepared in distilled water. Because edaravone and Trolox cannot be dissolved in distilled water, a 10 mM stock solution of edaravone was prepared in 0.01% H_2SO_4 solution and a 10 mM stock solution of Trolox was

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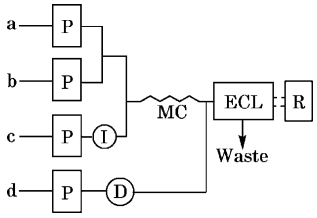

Fig. 1. A Schematic Diagram of FIA-ECL Detection

a, 3.0 mM H_2O_2 solution; flow rate, 1.2 ml/min. b, 0.75 mM $FeCl_2$ solution; flow rate, 1.0 ml/min. c, distilled water; flow rate, 0.8 ml/min. d, 0.5 mM $Ru(bpy)_3^{2+}$ in 5 mM H_2SO_4; flow rate, 0.4 ml/min. P, pump; I, injector (5 μl); ECL, ECL detector; R, recorder; D, dumper; MC, 10 cm mixing coil.

prepared in 10 mM NaOH solution. Determination of ·OH scavenging capacity was not influenced by 0.01% H_2SO_4 and 10 mM NaOH. Working solutions were prepared from stock solutions by dilution with distilled water.

Apparatus and Manifold Design The schematic diagram of the FIA-CL system is shown in Fig. 1. ·OH were obtained by the Fenton reaction. The flow rates of 3.0 mM H_2O_2 solution (a) and 0.75 mM $FeCl_2$ solution (b) were 1.2 ml/min with a uf-3005SZB2 pump (Uniflows, Tokyo, Japan) and 1.0 ml/min with a uf-4004p pump (Uniflows), respectively. The flow rate of the carrier solution (distilled water, c) was 0.8 ml/min with a Shimadzu LC-6A pump (Kyoto, Japan) equipped with a Reodyne 7125 sample injector (5 μl, Cototi, CA, U.S.A.). The reagent solution (d) used was 0.5 mM $Ru(bpy)_3^{2+}$ in 5 mM sulfuric acid solution, which was delivered at a flow rate of 0.4 ml/min with an Intelligent Pump 301 (Flow, Tokyo, Japan). The $FeCl_2$ solution, carrier solution, and reagent solution were pumped through a DG-2410 (Uniflows) as a degasser. Because $Ru(bpy)_3^{3+}$ in aqueous solution is unstable, $Ru(bpy)_3^{3+}$ has to be prepared from $Ru(bpy)_3^{2+}$ before use. Therefore, the $Ru(bpy)_3^{2+}$ solution was delivered and oxidized to $Ru(bpy)_3^{3+}$ by the controlled-current electrolysis method (Galvanostat Comet 2000, Comet, Kawasaki, Japan). The electrolytic current of the electrochemical reactor was set at 80 μA. A Pulse Dumper (Uniflows) was installed between the pump and the electrode cell to prevent pulsation due to the pump. $Ru(bpy)_3^{3+}$ and ·OH are in contact in the spiral cell in the detector, and thus, the chemiluminescence is constantly maintained. Chromatograms were recorded with Chromatopak C-R5A (Shimadzu, Kyoto, Japan). All experiments were performed at room temperature. A sludge is formed because of the Fenton reaction, and it is therefore necessary to flush the system with a 0.2 M sulfuric acid solution for about 20 min once every three hours. All connecting PTFE tube was 0.5 mm i.d.

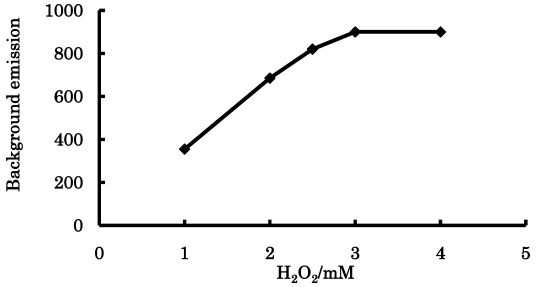

Fig. 2. Effect of H_2O_2 Concentration on the Background Emission

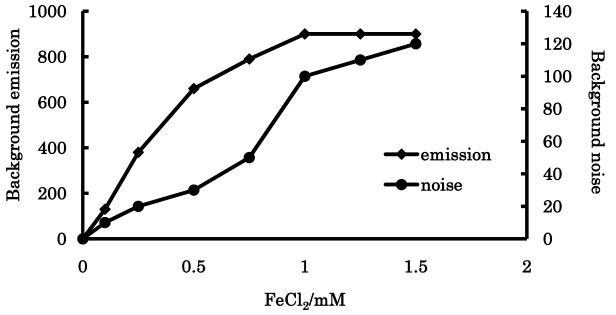

Fig. 3. Effect of $FeCl_2$ Concentration on the Background Emission

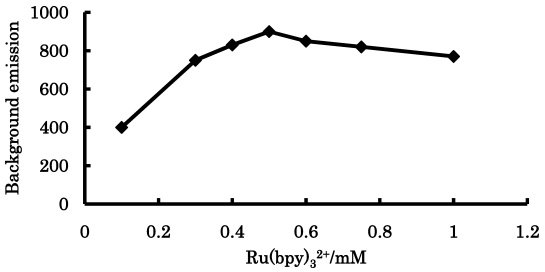

Fig. 4. Effect of $Ru(bpy)_3^{2+}$ Concentration on the Background Emission

Results and Discussion

Effect of H_2O_2 Concentration The effect of H_2O_2 concentration on the background emission is shown in Fig. 2. The amount of ·OH was directly influenced by H_2O_2 concentration. When no additional H_2O_2 is present, the background emission is zero. However, addition of extra H_2O_2 may accelerate the production of ·OH.[14,16] The results showed that the background emission increased with H_2O_2 concentration in the range of 1—3 mM and remained constant thereafter. Therefore, 3 mM concentration of H_2O_2 should be selected.

Effect of $FeCl_2$ Concentration The effect of $FeCl_2$ concentration on the background emission is shown in Fig. 3. The background emission increased with $FeCl_2$ concentration in the range of 0.1—1.0 mM and remained constant when the concentration was above 1.0 mM. The background emission increased with increasing $FeCl_2$ concentration, but values higher than 0.75 mM $FeCl_2$ are not recommended because background noise increased as well. Therefore, 0.75 mM concentration of $FeCl_2$ should be selected.

Effect of $Ru(bpy)_3^{2+}$ Concentration It is suggested that ·OH generates excited $Ru(bpy)_3^{2+}$* in an electron transfer reaction with $Ru(bpy)_3^{3+}$. ·OH and $Ru(bpy)_3^{3+}$ are in contact in the spiral cell in the detector, and chemiluminescence is continuously maintained. The effect of the concentration of $Ru(bpy)_3^{2+}$ on the background emission is shown in Fig. 4. The background emission increased with $Ru(bpy)_3^{2+}$ concentration in the range of 0.1—0.5 mM and slightly decreased when the concentration of $Ru(bpy)_3^{2+}$ was above 0.5 mM. Therefore, 0.5 mM $Ru(bpy)_3^{2+}$ in 5 mM H_2SO_4 solution was chosen as the optimum concentration. From the results of the optimization study, 3.0 mM H_2O_2 solution, 0.75 mM $FeCl_2$ solution, and 0.5 mM $Ru(bpy)_3^{2+}$ in 5 mM H_2SO_4 solution were selected for the determination of ·OH scavenging capacity.

Effect of Flow Rates and Mixing Coil Length The flow rate was an important factor for sample throughput and baseline stability. With lower flow rates of H_2O_2 solution and $FeCl_2$ solution, the background emission decreased because of a decrease in the reaction rate. The flow rates of H_2O_2, $FeCl_2$, carrier, and reagent solutions were 1.2 ml/min, 1.0 ml/min, 0.8 ml/min, and 0.4 ml/min, respectively. These flow rates were selected in order to maintain baseline stability, ensure sensitivity, and increase sample throughput. However, a higher flow rate should decrease the residence time of the scavenging sample and ·OH in the mixing coil, thus reducing the reaction time. Therefore, a 10-cm mixing coil was chosen.

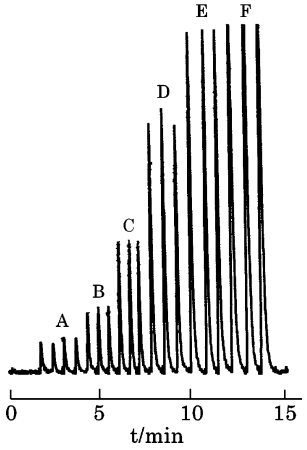

Fig. 5. Typical Response of Trolox Obtained by FIA-ECL Detection

A: 0.25 mM; B: 0.5 mM; C: 1.0 mM; D: 2.5 mM; E: 5 mM; F: 10 mM. FIA conditions are the same as in Fig. 1.

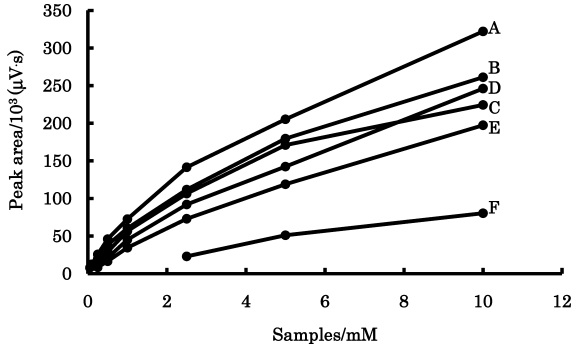

Fig. 6. ·OH Scavenging Capacity by Edaravone (A), L-Tryptophan (B), Gallic Acid (C), Trolox (D), *N*-Acetyl-L-cysteine (E), and Ascorbic Acid (F)

FIA conditions are the same as in Fig. 1.

·OH Scavenging Capacity When $Ru(bpy)_3^{3+}$ and ·OH are in contact, chemiluminescence is continuously maintained. ·OH was obtained from the Fenton reaction. Constant chemiluminescence is generated and recorded as the background emission. The background emission decreases in proportion to the sample concentration when scavenging samples are injected. Edaravone, L-tryptophan, gallic acid, Trolox, *N*-acetyl-L-cysteine, and ascorbic acid are commonly used as ·OH scavenging samples.[10,13,21,22] Figure 5 shows a typical FIA profile obtained from Trolox. As shown in Fig. 6, the six samples had good scavenging capacity, which decreased in the following order: edaravone>L-tryptophan>gallic acid>Trolox>*N*-acetyl-L-cysteine>ascorbic acid. Edaravone had the best ·OH scavenging capacity under our optimum conditions. The ·OH scavenging capacity (2.5 mM concentrations of each) of edaravone was 21%, 33%, 55%, 100%, and 530% higher than that of L-tryptophan, gallic acid, Trolox, *N*-acetyl-L-cysteine, and ascorbic acid, respectively (Fig. 7). This method allowed a sample throughput of about 80 samples/h.

Relative Standard Deviation and Lower Limit of Quantification The samples were measured 10 times (n=10). The relative standard deviations (RSDs) for determination of edaravone, L-tryptophan, gallic acid, Trolox, *N*-acetyl-L-cysteine, and ascorbic acid were 3.35% (1.0 mM), 3.24% (1.0 mM), 3.52% (1.0 mM), 3.91% (1.0 mM), 3.74% (1.0 mM), and 3.95% (10.0 mM), respectively, and their lower limits of quantification were 0.05 mM (n=10, RSD=5.13%), 0.1 mM (n=10, RSD=6.52%), 0.25 mM (n=10, RSD=5.52%), 0.25 mM (n=10, RSD=13.9%), 0.25 mM (n=10, RSD=10.6%), and 2.5 mM (n=10, RSD=7.66%), respectively. These results indicated that our proposed method can be used to evaluate antioxidant capacity against ·OH.

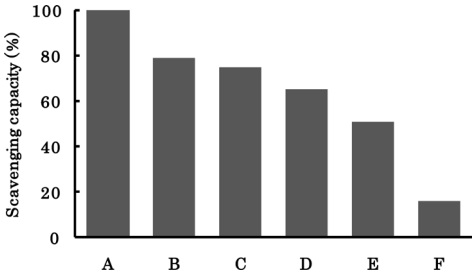

Fig. 7. ·OH Scavenging Capacity of Edaravone (A), L-Tryptophan (B), Gallic Acid (C), Trolox (D), *N*-Acetyl-L-cysteine (E), and Ascorbic Acid (F) (2.5 mM Each) against ·OH

FIA conditions are the same as in Fig. 1.

Conclusion

We have clearly shown here that ·OH generates excited $Ru(bpy)_3^{2+}$* in an electron transfer reaction with $Ru(bpy)_3^{3+}$, and that the background emission is generated. ·OH is generated by the Fenton reaction. Edaravone had the best ·OH scavenging capacity under our experimental conditions. The proposed method is a rapid, selective, and accurate procedure for the study of ·OH scavenging capacity based on $Ru(bpy)_3^{3+}$ chemiluminescence by flow injection analysis.

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