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Quantitative Analysis of Hydroperoxyl Radical Using Flow Injection Analysis with Chemiluminescence Detection

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The hydroperoxyl radical (HO_2) is one of the most abundant free radicals in the atmosphere, where it participates in a series of photochemical reactions that determine the fate of natural and anthropogenic emissions. In addition, HO_2 is found in droplets and surface water as a result of photochemical formation and gas-phase scavenging. We describe a quantitative method for determining trace concentrations of HO_2 radicals that exploits the chemiluminescence produced upon reaction with a synthetic analogue of luciferin from the crustacean *Cypridina*. The technique is linear at least up to 1 μM $HO_2(aq)$ and has a minimum detection limit of 0.1 nM. A unique feature of this analysis is a calibration method using stable aqueous HO_2 standards produced in submicromolar concentrations using ^{60}Co γ-radiolysis. The advantage of this method in comparison to others in consideration of field deployment is its simplicity, low cost, and minimal size and power requirements. One intended application of this technique is the measurement of atmospheric HO_2 radicals following collection into aqueous solution.

The hydroperoxyl radical (HO_2) is one of the most abundant free radicals in the atmosphere. HO_2 plays an important role in urban smog formation through its reaction with NO to yield NO_2, which is photolyzed in the troposphere to generate O_3

$$HO_2 + NO \rightarrow NO_2 + OH \qquad (R1)$$

$$NO_2 \xrightarrow{\lambda < 420 \text{ nm}} NO + O \qquad (R2)$$

$$O + O_2 \rightarrow O_3 \qquad (R3)$$
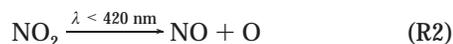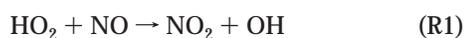
Peroxy radicals in the atmosphere originate from reaction of the hydroxyl radical with organic compounds in the presence of oxygen. Reaction of OH with CO forms HO_2 directly (R4), while analogous reactions with other organic compounds yield alkyl-peroxy radicals, RO_2 via R5

$$OH + CO \xrightarrow{O_2} CO_2 + HO_2 \qquad (R4)$$

$$OH + RH \xrightarrow{O_2} RO_2 + H_2O \qquad (R5)$$

Except in the remote troposphere, the fate of RO_2 is dictated by its reaction with NO to produce NO_2 and alkoxy radicals. Some alkoxy radicals subsequently may react to produce additional HO_2.[1]

Atmospheric HO_2 is highly soluble[2] and will partition from air into an available aqueous phase, where it equilibrates rapidly with its conjugate base, superoxide anion, $O_2^-(aq)$:

$$HO_2 \leftrightarrow H^+ + O_2^- \qquad (R6)$$

Photochemical formation is another source of these radicals in surface waters and droplets.[3] HO_2 has a pK_a of 4.8, and the acid and base forms generally exhibit very different aqueous-phase reactivities.[4] Hereinafter, we will refer to the equilibrium mixture of HO_2 and O_2^- in aqueous solution as $HO_2(aq)$; this term refers to the proper apportionment of HO_2 and O_2^- at the pH in question.

The low atmospheric abundance and high reactivity of HO_2 radicals, combined with the lack of a strong unique spectral

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signature or quantitative standards, presents an analytical challenge. Nevertheless, the past decade has seen the emergence of several sensitive, fast, and highly specific methods for the detection of these species in the atmosphere. The peroxy radical chemical amplifier method, developed by Cantrell et al.,[5] produces OH and NO_2 by the reaction of excess NO with HO_2 (R1). Excess CO is added to recycle OH back to HO_2 (R4), and the resulting chain-amplified NO_2 signal is measured by luminol chemiluminescence. This method has a 1 pptv detection limit but is subject to interference from atmospheric O_3, PAN, and NO_2. Determination of the chain length may introduce a significant error into the measurement.[6] Mihelcic et al. have reported peroxy radical abundances using ESR spectroscopy after trapping the radicals in a D_2O matrix at 77 K.[7,8] These investigators use a numerical fitting procedure that permits the determination of HO_2 in the presence of RO_2 with a detection limit of 5 pptv for a 30-min sampling interval. HO_2 also can be measured after reaction with excess NO, followed by detection of the resulting OH by laser-induced fluorescence (LIF) at 308 nm.[9,10] LIF is highly specific for OH and exhibits a 0.03 pptv detection limit. A peroxy radical chemical ionization mass spectroscopy (PerCIMS) technique has been developed for the measurement of tropospheric HO_2 and RO_2.[11] For this measurement, excess NO is added to produce OH upon reaction with peroxy radicals. Hydroxyl radicals react rapidly with added isotopically labeled SO_2, and the resulting labeled sulfuric acid is detected by quadrupole mass spectrometry. PerCIMS has excellent sensitivity and may be able to discriminate between HO_2 and RO_2 by controlling the concentration of added NO and SO_2.

All of the methods described above have been deployed in recent field programs with varying degrees of success and are still very much under development. We describe here a continuous, fast, and specific HO_2 detector that is based on the chemiluminescent reaction between HO_2(aq) and a synthetic analogue of the luciferin from the crustacean *Cypridina*.

Biochemists have reported that certain proteins extracted from marine animals luminesce in the presence of activated granulocytes (e.g., refs 12 and 13). Emission of light from synthetic *Cypridina* luciferin analogue (CLA) in the superoxide-generating xanthine/xanthine oxidase system prompted early suggestions that HO_2(aq) was involved in the reaction.[14] This was supported

by the observation that the enzyme superoxide dismutase suppressed luminescence. The addition of mannitol, which scavenges hydroxyl radical, did not inhibit luminescence, nor did azide, which scavenges $^1O_2$, nor did catalase, which destroys peroxide. CLA luminescence is now widely used to demonstrate the putative involvement of HO_2(aq) in biological processes. A mechanism for reaction of CLA with HO_2 has been proposed (R7), and the pH

R7
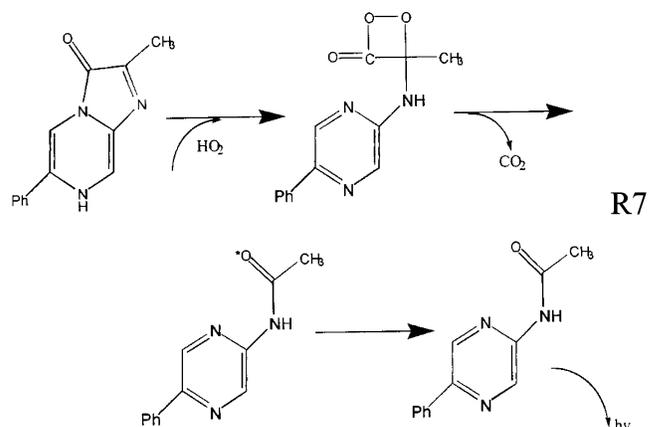
dependence of the reaction rate has been evaluated.[15] The chemiluminescence spectrum depends on pH, peaking at 380 nm at pH 7.0 and 450 nm at pH 11.1.[16] A growing literature now provides reaction rates of HO_2(aq) with various compounds measured by competition kinetics, using the CLA + HO_2(aq) rate constant as a reference.[17] A number of CLA analogues have been synthesized; these vary in aqueous solubility, sensitivity to HO_2, stability versus pH, and background emission.[18]

We are investigating this family of chemiluminescence reagents for the quantitative analysis of HO_2. Previous investigations in biological systems have focused on the rate of generation of HO_2(aq), rather than the absolute concentration, because enzymatic radical generation systems produce variable quantities of HO_2(aq). Rather than using complex biological systems and their associated buffers, we produce known quantities of HO_2(aq) in aqueous solution for calibration standards using ^{60}Co γ-radiolysis. We take advantage of the elegant work of Bielski and Arudi,[19] who showed that it is possible to produce stable solutions of O_2^- at high pH. We have demonstrated that CLA and MCLA emit light upon reaction with radiolytically produced HO_2(aq) radicals and have examined potential interferences as a function of pH. We have incorporated the chemiluminescent HO_2(aq) + CLA chemistry into a flow injection analysis (FIA) apparatus similar to that used for determination of atmospheric hydroperoxides[20] and have shown it to be linear and sensitive to aqueous HO_2(aq). This paper describes the experimental apparatus and optimum conditions for the analysis of HO_2(aq) solutions.

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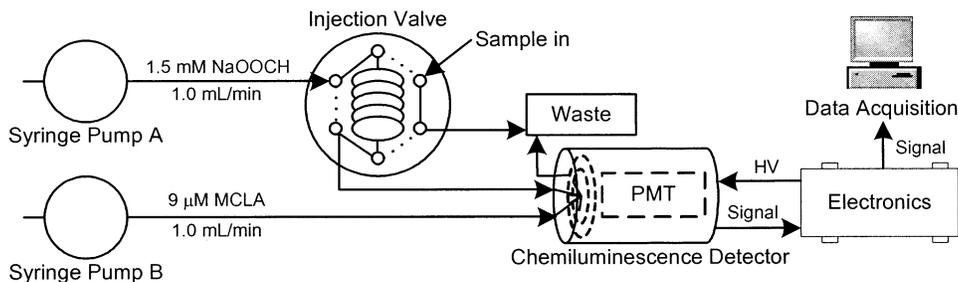
Figure 1. Schematic diagram of the FIA-based HO$_2$(aq) chemiluminescence detector.

EXPERIMENTAL SECTION

Reagents. All reagents used were the purest grade available, and all solutions were prepared using 18.2-MΩ water purified by a Millipore system (Billerica, MA). The *Cypridina* luciferin analogues 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (CLA) and 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo-[1,2-*a*]pyrazin-3-one (MCLA) were purchased from TCI America (Portland, OR) and used without purification. Sodium formate (Baker, Analyzed Reagent Grade) and sodium phosphate (GFS Chemicals) for radiolysis experiments were recrystallized twice, first from Millipore water containing 10^{-4} M EDTA to remove trace metal impurities and then from Millipore water alone. Sodium hydroxide (GFS Chemicals, 99.999%) and hydrochloric acid (Sigma Co., 99.999%) were used without further purification. Interference studies were conducted using H$_2$O$_2$ (3%, Mallinck-rodt) and O$_3$ (Thermo Environmental model 165C ozone generator).

Aqueous Standards. O$_2^-$ stock solutions were prepared by ^{60}Co γ-irradiation of air-saturated aqueous solutions containing 1.5 mM sodium formate and 5 μM EDTA, adjusted to pH 11 with NaOH. Under these conditions, the primary radicals H, OH, and e$_{aq}^-$ are converted to an equilibrium mixture of O$_2^-$ and HO$_2$ within milliseconds.[21] In the absence of additives, O$_2^-$ and HO$_2$ radicals decay by self-reactions R8—R10 with a pH-dependent rate coefficient (k_{obs}):

$$HO_2 + HO_2 \rightarrow H_2O_2 + O_2 \qquad (R8)$$

$$HO_2 + O_2^- + H_2O \rightarrow H_2O_2 + O_2 + OH^- \qquad (R9)$$

$$O_2^- + O_2^- + 2H_2O \rightarrow H_2O_2 + O_2 + 2OH^- \qquad (R10)$$
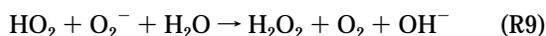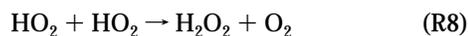
$$k_{obs} = \frac{k_8 + k_9(K_6/[H^+])}{(1 + K_6/[H^+])^2}$$

where $k_8 = 8.3 \times 10^5$, $k_9 = 9.7 \times 10^7$, and $k_{10} < 0.3$ L mol^{-1} s^{-1}.[4] ^{60}Co source intensity, measured by ferrous sulfate dosimetry, was 2.77 rads min^{-1}, producing 0.290 μM HO$_2$(aq) radicals min^{-1}. At pH 11, a sample initially containing 1 μM O$_2^-$ has a half-life of 2.8 h. We verify O$_2^-$ concentration spectrophotometrically ($\epsilon_{240} = 2300$ L mol^{-1} cm^{-1}), immediately dilute the sample by a factor of 100, and store it at 0 °C. Calibrations are conducted within 8 h using this diluted solution, which has an O$_2^-$ half-life on the order of days.

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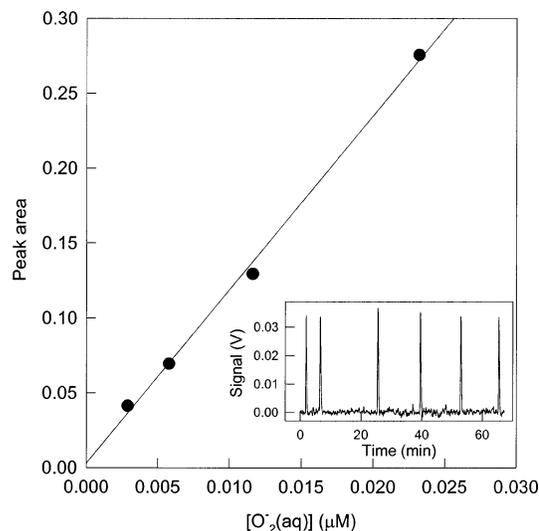
Figure 2. Calibration of the HO$_2$(aq) detector using radiolytic standards. Standards are air-saturated and contain 1.0 mM NaOOCH adjusted with NaOH to pH 11, irradiated by ^{60}Co for 1.0 min, and then diluted 1:100 into unirradiated formate solution. Inset shows stability of HO$_2$(aq) standards (peak area 0.732 $\pm$ 0.022). Standards contain 8.7 nM O$_2^-$(aq), 5.0 μM MCLA, sample volume 205 μL, and total flow rate 1.50 mL/min.

Figure 1 is a schematic diagram of the apparatus. Two high-precision syringe pumps (model 260D, Isco Inc., Lincoln, NE) deliver a reagent stream and a carrier stream to a chemiluminescence detector (model 660, McPherson Inc., Chelmsford, MA) at 1.0 mL min^{-1}. The carrier stream consists of unirradiated formate solution adjusted to pH 11 with NaOH, and the reagent stream is a 9 μM MCLA solution in 1:100 (v/v) ethanol/H$_2$O, adjusted to pH 2.3 with HCl. HO$_2$(aq) samples are injected into the carrier stream using a six-port injection valve (VICI, Houston, TX). The chemiluminescence detector has a 500-μL spiral reaction cell that permits reagents to mix directly in front of a Hamamatsu R-268 photomultiplier tube.

RESULTS AND DISCUSSION

Calibration. Figure 2 is a calibration curve using radiolytically produced standards, with an inset that shows that the standards are stable for more than 1 h, substantially longer than necessary for calibration. The pH of the mixed carrier and reagent stream is 3. At this pH, the half-life of HO$_2$(aq) with respect to reaction with MCLA is 1 s,[15] while the self-reaction has a half-life of 30 s. Thus, there is no significant loss of HO$_2$(aq) to self-reaction during the calibration. The baseline-to-baseline width of the FIA signal

Figure 3. Determination of optimum conditions for detection of 2.9 nM $HO_2(aq)$. Effect of (a) flow rate for 205-μL injected sample volume, [MCLA] = 10 μM; (b) sample size for flow rate 0.5 mL min^{-1}, [MCLA] = 10 μM; (c) MCLA concentration for 80-μL injected volume at 1.0 mL min^{-1} flow rate.

is 50 s, which allows for an ~1-min sampling frequency for continuous measurements. The calibration is linear, with a detection limit of 0.12 nM, based on three times the baseline noise.

Optimization and Interferences. We varied chemical composition, flow rates, and sampling volume to determine the optimum reaction conditions. Signal area decreased with solution flow rates up to 1.0 mL min^{-1} and became constant (Figure 3a). Peak area showed a maximum for an injected volume of 200 μL of $HO_2(aq)$ (Figure 3b). An MCLA concentration of 12 μM produced the largest response (Figure 3c).

Samples of H_2O_2 and O_3 were tested to see if they generated a luminescence response from MCLA. Addition of 1 μM H_2O_2 generated a luminescence signal equivalent to 0.1 nM $HO_2(aq)$, but we observed no response from an aqueous sample that had been saturated with a gaseous stream containing 150 ppbv O_3 in O_2.

It is worth noting that air oxidation of MCLA occurs at high pH, producing luminescence identical to that from HO_2,[16] but the HO_2 standards must be kept at high pH to avoid the fast reaction R9. Thus, adjusting the pH to maintain the titer of both MCLA and HO_2 standards is critical.

DISCUSSION

We have demonstrated that MCLA can be used for the routine determination of $HO_2(aq)$ in aqueous solution using FIA, with a detection limit of 1×10^{-10} M. Using ^{60}Co γ-radiolysis to generate $HO_2(aq)$ samples, we have verified the fact that these radicals react with MCLA to generate luminescence. We have demonstrated that radiolytically produced $HO_2(aq)$ samples can be used as calibration standards and have determined conditions for optimum detection. There are no published reports of the quantitative application of this technique to HO_2 radical detection (see, for example, a review of analytical applications of flow injection with chemiluminescence detection[22]). For atmospheric applications, we plan to collect HO_2 using a high-pH stripping solution. Although the published Henry's law constant for HO_2 varies from 4×10^3 to 9×10^3 M atm^{-1},[23] its large magnitude causes the collection efficiency to be rather insensitive to the exact value. For a liquid flow rate of 1 mL min^{-1} and an air flow rate of 1 L min^{-1}, calculated collection efficiencies are 98.9 and 99.5%,

Table 1. Potentially Interfering Gaseous Oxidants

species	k_H (M/atm)[a]	atmos abund (ppb)[b]	collection efficiency[c]	expected concn (M)
H_2O_2	7.4×10^4	5	99	4.0×10^{-7}
O_3	1.1×10^{-2}	100	1.2×10^{-4}	9.7×10^{-10}
HO_2	9×10^3	0.050	99	4.0×10^{-9}
OH	25	0.0005	0.22	8.7×10^{-12}
$^1O_2$	1.3×10^{-3d}	0.005	1.5×10^{-5}	6.1×10^{-15}

[a] Reference 23. [b] Reference 28. [c] Based on 1 mL min^{-1} liquid flow and 1 L min^{-1} air flow in glass scrubbing coil. [d] Assumed to be the same as O_2.

respectively, for the high and low value of K_H. High-pH stripping solution will favor the dissociation of HO_2 to O_2^-, increasing the effective Henry's law constant and further reducing the uncertainty in collection efficiency. These flow rates should result in a gas-phase detection limit of 1 pptv HO_2, adequate sensitivity for polluted areas where midday concentrations of HO_2 are expected to peak near 100 pptv.[24]

Several investigators have published rate constants for the reaction between O_2^- + CLA, all using enzymatically produced O_2^-. Two of these values, 2.12×10^5 M^{-1} s^{-1} at pH 7.8[25] and 6.7 $\times 10^4$ M^{-1} s^{-1} at pH 8.2[15] are of similar magnitude, while one, 1.08×10^8 M^{-1} s^{-1} at pH 7.0,[26] is very much higher. Reliable determination of the relevant pH-dependent rate constant is important, as the rate of the CLA + O_2^- reaction is now being used as a standard for competition kinetics for reactions of O_2^- with a variety of species.[17] We are conducting a systematic pulse radiolysis study of the kinetics of reaction of the CLA family with $HO_2(aq)$; results of this study will be reported elsewhere.

Because of the high sensitivity of the chemiluminescence method, it is critical to evaluate potential interferences from atmospheric oxidants that may be co-collected in the stripping coil. These species and their Henry's law solubilities are given in Table 1, together with ambient gas-phase abundances and the resulting aqueous concentrations expected in our apparatus under typical operating conditions. H_2O_2 is the most abundant water-soluble atmospheric oxidant and may be collected in the gas—aqueous scrubbing coil at concentrations in the micromolar

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ange.[20] MCLA was reported to be unreactive toward H_2O_2 based on the inability of catalase enzyme to quench HO_2-generated luminescence.[14] As discussed earlier, we have confirmed that luminescence from reaction with H_2O_2 is minimal, but we are seeking experimental conditions that will reduce this interference. Although much less soluble than H_2O_2, O_3 may be 100 times more abundant; absence of O_3 interference was therefore important to substantiate. Reactions with OH and $^1O_2$ are not expected to be a problem due to their extremely low aqueous-phase concentrations but will be explored in future work.

Loss of sample in inlet tubing is an important concern for HO_2 radicals due to their labile nature and high solubility in surface-adsorbed water. We plan to mitigate this problem with an "inletless" glass coil collection device that directs sampled air into an aqueous stripping solution through a 1-mm orifice.[27] This method prevents contact of gaseous HO_2 radicals with the wall.

In addition to kinetic studies of HO_2(aq) with MCLA and related chemiluminescence reagents, future work will focus on integrating the FIA detection system with a gas-phase sample collection apparatus. One important area of investigation will be the extent to which the CLAs respond to organic peroxy radicals, e.g., CH_3O_2 and $C_2H_5O_2$, which are expected to be abundant in the atmosphere. These radicals are less water soluble than HO_2 and, in aqueous solution, are unlikely to form the dioxetane intermediate responsible for light emission, suggesting that they may not interfere. Studies of other potentially interfering species are ongoing.

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