Quantification of nanomolar superoxide in aqueous solution: flow injection analysis using the chemiluminescent reagent MCLA.

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Quantification of nanomolar superoxide

in aqueous solution:

Flow injection analysis using the chemiluminescent reagent MCLA

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May 11, 2001
Quantification of nanomolar superoxide in aqueous solution:

Flow injection analysis using the chemiluminescent reagent MCLA

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May 11, 2001

Submitted in partial fulfillment of the Requirements for Honors in Chemistry

Approved

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Abstract

A flow injection system using the chemiluminescent reagent 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (MCLA) was used to quantify aqueous concentrations of superoxide. Stock solutions of superoxide were produced by photolysis of a solution of 2-propanol and benzophenone. The concentration of superoxide was quantified in the stock solutions by direct measurement of the absorbance at 240 nm. Quantified additions of superoxide to the flow injection analysis system reacted with MCLA at pH 5.1 demonstrated a detection limit of 0.074 nM superoxide. Selective superoxide detection was confirmed by pH-dependent decay of superoxide, which was in excellent agreement with published rates.
Introdução

Superoxide, NO₂ and its conjugate base O₂⁻, are reactive oxygen species produced by the one electron reduction or oxidation of molecular oxygen or hydrogen peroxide, respectively. The major source of superoxide in natural aquatic systems is from sunlight photolysis of colored dissolved organic matter (CDOM) (eqns. 1 and 2).

\[
\text{CDOM} + \text{hv} \rightarrow \text{CDOM}^* \quad (1)
\]

\[
\text{CDOM}^* + \text{O}_2 \rightarrow \text{CDOM}^+ + \text{O}_2^- \quad (2)
\]

It has been suggested that direct electron transfer, intramolecular electron transfer, H-atom abstractions, or homolytic bond cleavages are responsible for the generation of intermediate radical oxygen species (ROS) that then react to produce superoxide.¹ Minor sources of superoxide include production by reactions of transition metals with dissolved oxygen (eqn. 3).² Superoxide can be biologically produced by mitochondria as a metabolic side reaction of the reduction of oxygen.

\[
\text{O}_2 + \text{L}_m\text{M}^{n+} \rightleftharpoons \text{L}_m\text{MO}_2^{n+} \rightleftharpoons \text{O}_2^- + \text{L}_m\text{M}^{(n+1)} \quad (3)^2
\]

The total superoxide is the sum of all protonated and unprotonated superoxide in solution as determined by the dissociation reaction (eqn. 4).

\[
\text{HO}_2 \rightleftharpoons \text{H}^+ + \text{O}_2^- \quad (4)^\dagger
\]

In pure water systems, total superoxide decays by the disproportionation reactions 5 and 6 (reactions of O₂⁻ with itself are negligible due to charge repulsion).

\[
\text{HO}_2 + \text{HO}_2 \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (5)^\dagger
\]

\[
\text{HO}_2 + \text{O}_2^- \rightarrow \text{HO}_2^- + \text{O}_2 \quad (6)^\dagger
\]

^† rate constants are available in Table I.
Table I. Reaction Rates

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Reaction Equation</th>
<th>Rate Constants(M^{-1}s^{-1})</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>H^+ + O_2^- → HO_2</td>
<td>1.60E-05</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>HO_2 + HO_2 → H_2O_2 + O_2</td>
<td>8.30E+05</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>HO_2 + O_2^- → HO_2^- + O_2</td>
<td>9.70E+07</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>Fe^{II} + HO_2 + H^+ → Fe^{III} + H_2O_2</td>
<td>1.20E+06</td>
<td>21</td>
</tr>
<tr>
<td>11</td>
<td>Fe^{II} + O_2^- + H^+ → Fe^{III} + HO_2^-</td>
<td>1.00E+07</td>
<td>21</td>
</tr>
<tr>
<td>12</td>
<td>Fe^{III} + O_2^- → Fe^{II} + O_2</td>
<td>1.50E+08</td>
<td>21</td>
</tr>
<tr>
<td>13</td>
<td>Fe^{III} + HO_2 → Fe^{II} + H^+ + O_2</td>
<td>&lt;1.00E+03</td>
<td>21</td>
</tr>
<tr>
<td>14</td>
<td>Cu^{I} + O_2^- + 2H^+ → Cu^{II} + H_2O_2</td>
<td>1.00E+09</td>
<td>24</td>
</tr>
<tr>
<td>15</td>
<td>O_2^- + Cu^{I} + H_2O → Cu^{II} + OH^- + HO_2^-</td>
<td>1.00E+10</td>
<td>24</td>
</tr>
<tr>
<td>16</td>
<td>HO_2 + Cu^{I} → H^+ + Cu^{I} + O_2</td>
<td>1.00E+08</td>
<td>24</td>
</tr>
<tr>
<td>17</td>
<td>Cu^{II} + O_2^- → Cu^{I} + O_2</td>
<td>5.80E+09</td>
<td>24</td>
</tr>
</tbody>
</table>
The total superoxide decay rate constant is the sum of the rate constants for equations 5 and 6, which is dependent upon pH.

The pH dependence of the observed decay constant, $k_{obs}$, is given by the partition function for $\text{HO}_2^-$ (eqn. 7) and $\text{O}_2^-$ (eqn. 8) times the rate constants for reactions 5 and 6.

$$
\alpha_0 = \frac{H^+}{(H^+ + K_4)} \\
\alpha_1 = \frac{K_4}{(H^+ + K_4)} \\
k_{obs} = \alpha_0 k_5 + \alpha_1 k_6
$$

The plot of $k_{obs}$ vs. pH (fig. 1) has a characteristic shape. For pHs $> 6$ the curve has a slope of $-1$. For pHs $< 2$ the decay rate is constant. Between pHs of 2 and 6 reactions 5 and 6 are both contributing to the rate with a maximum at the pK$_a$, $K_a$, of $\text{O}_2^-$. In pure waters the principal sink of superoxide is the production of hydrogen peroxide through spontaneous disproportionation, eqs. 5 and 6. Superoxide is also important in the redox cycling of dissolved trace metals (eqns. 10-17)\textsuperscript{,**,4,5}

$$
\begin{align*}
\text{Fe}^{II} + \text{O}_2^- + H^+ & \rightarrow \text{Fe}^{III} + \text{HO}_2^- \\
\text{Fe}^{II} + \text{HO}_2 + H^+ & \rightarrow \text{Fe}^{III} + H_2O_2 \\
\text{Fe}^{III} + \text{O}_2^- & \rightarrow \text{Fe}^{II} + O_2 \\
\text{Fe}^{III} + \text{HO}_2 & \rightarrow \text{Fe}^{II} + H^+ + O_2 \\
\text{Cu}^{I} + \text{O}_2^- + 2 H^+ & \rightarrow \text{Cu}^{II} + H_2O_2 \\
\text{Cu}^{I} + \text{O}_2^- + H_2O & \rightarrow \text{Cu}^{II} + OH^- + \text{HO}_2^- \\
\text{Cu}^{II} + \text{HO}_2 & \rightarrow \text{Cu}^{I} + H^+ + O_2 \\
\text{Cu}^{II} + \text{O}_2^- & \rightarrow \text{Cu}^{I} + O_2 \\
\end{align*}
$$

Reactions with the aqua-complexes of dissolved metals

\textsuperscript{†}Rate constants are available in Table I.
Figure 1. Second order decay rate constant for HO₂ / O₂⁻ vs. pH (adapted from ref. 3).
[O_2] flux. However, studies by Beckman et. al. and Yang et. al. suggest that other paths for NO rearrangement might cause this method to underestimate superoxide production.\textsuperscript{10,11} In addition, NO methods require intensive, daylong sample processing.\textsuperscript{12} Recently, 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (MCLA) (shown below), an analogue of Cypridina Luciferin, has been used to detect superoxide in biological systems.\textsuperscript{13-15} Asai reported a MCLA chemiluminescence (CL)-based superoxide flow injection analysis (FIA) system for quantification of \textit{C. antiqua}.\textsuperscript{16} However, the system was not calibrated for the quantitative measurement of superoxide.

\begin{center}
\includegraphics[width=0.5\textwidth]{mcla.png}
\end{center}

\textit{Superoxide Standards}. Direct studies of aqueous superoxide quantification are difficult because of its reactivity. In order to quantify the superoxide detection system, a clean, stable and quantifiable stock of superoxide was needed. KO_2 is a readily available source, but is commercially available at only about 96\% purity and is full of peroxides and metal contaminants.\textsuperscript{2} Pulse radiolysis is most frequently used for superoxide production,\textsuperscript{17-20} especially in kinetics experiments, but the method is equipment intensive. An inexpensive alternative has been developed by McDowell et. al. based on the indirect production of superoxide through the photolysis of a ketone (eqn. 18).\textsuperscript{21} The photolysis of a ketone in the presence of a secondary or tertiary alcohol catalytically forms two superoxide radicals a new ketone product. The method has been successfully used by Goldstone et. al. in their kinetic studies of CDOM and Cu reactions with superoxide in natural waters.\textsuperscript{5}
Spectral Analysis of Superoxide. The protonated and unprotonated forms of superoxide have different UV absorption spectra.\(^3\) Table II contains the tabulated pH dependent total molar extinction coefficients superoxide. However, these values are only relevant for superoxide before it undergoes spontaneous disproportionation via reactions 5 and 6 to form \(\text{H}_2\text{O}_2\), which also absorbs light in the same region as superoxide. Thus, any measurements of superoxide undergoing disproportionation requires correction for the production of \(\text{H}_2\text{O}_2\).

This work describes a quantitative method for \(\text{O}_2^-\) analysis using FIA-CL and employing MCLA as the analytical reagent.
Table II. Total Molar Absorptivity of Superoxide as a Function of pH

<table>
<thead>
<tr>
<th>pH</th>
<th>$\varepsilon$ 230 nm</th>
<th>$\varepsilon$ 240 nm</th>
<th>$\varepsilon$ 250 nm</th>
<th>$\varepsilon$ 260 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 - 1.5</td>
<td>1400</td>
<td>1260</td>
<td>915</td>
<td>540</td>
</tr>
<tr>
<td>2.0</td>
<td>1401</td>
<td>1261</td>
<td>917</td>
<td>542</td>
</tr>
<tr>
<td>2.5</td>
<td>1404</td>
<td>1265</td>
<td>922</td>
<td>547</td>
</tr>
<tr>
<td>3.0</td>
<td>1413</td>
<td>1277</td>
<td>936</td>
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</tr>
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<td>3.5</td>
<td>1440</td>
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<td>669*</td>
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<td>4.0</td>
<td>1514</td>
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<td>1101</td>
<td>733</td>
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<td>1679</td>
<td>1624</td>
<td>1366</td>
<td>1010</td>
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<td>1911</td>
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<td>6.5</td>
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</tr>
<tr>
<td>7.5</td>
<td>2228</td>
<td>2342</td>
<td>2257</td>
<td>1937</td>
</tr>
<tr>
<td>8.0 - 13.0</td>
<td>2230</td>
<td>2345</td>
<td>2260</td>
<td>1940</td>
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</table>

* corrected term

<table>
<thead>
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<th>Species</th>
<th>$\varepsilon$ 240 nm</th>
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<tr>
<td>$H_2O_2$</td>
<td>31</td>
</tr>
<tr>
<td>$HO_2^-$</td>
<td>320</td>
</tr>
</tbody>
</table>
Experimental

Superoxide Stock Solutions. We implemented McDowells photolysis method\textsuperscript{21} using a flow system, affectionately christened the "Juicer" (fig 2). The solution used in the Juicer, or "Juice," was made with benzophenone (0.63 µM) and 2-propanol (0.5 M) in a dilute alkaline buffer. Photolysis of the Juice, referenced with non-photolyzed Juice, showed characteristic spectra with absorbance maxima at 240 nm (fig. 3). After initial photolysis, the absorbance of superoxide at 240 nm decayed due to loss of superoxide and the production of the more weakly absorbing hydrogen peroxide. Both the decay of superoxide and the production of hydrogen peroxide were quantified in our studies. Fresh (non-photolyzed) Juice was used for each photolysis.

Flow Injection System. A FIA system (fig. 4) was used to inject a superoxide sample into a CL flow cell where it reacted with the CL probe MCLA, buffered with acetate, to produce light.\textsuperscript{22,23} The carrier was 18 MΩ H₂O. The sample was injected every 90 sec and data were collected for 50 sec at 3 pts/sec. The quantified CL signal was determined by integration of the light flux as a function of time.

Reagents. The chemiluminescent reagent 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, hydrochloride (MCLA) was purchased from Molecular Probes. Trace metal grade NaOH, benzophenone, 2-propanol, concentrated HCl, and sodium acetate were purchased from Fisher Scientific. Diethylenetriamine pentacetic acid, anhydride (DTPA) and tris[hydroxymethyl]aminomethane (Tris) were purchased from Sigma. All reagents were reagent grade unless otherwise specified and were used as received.
Reactions of superoxide with CDOM are also a major sink. These rates may be faster than disproportionation rates in many natural water types.

Consequently, the steady state concentrations of superoxide in lake or ocean water are expected to be controlled by the CDOM and dissolved metal sinks. The interplay of these processes is thought to produce steady state concentrations around $10^{-10}$ to $10^{-11}$ M in near-surface waters, but these concentrations have not been directly measured because direct quantitative analysis has not been available.

The many production and destruction reactions of superoxide have a significant impact on the oxidation of biological materials, and the oxidation/reduction of metal ions and organic compounds. The ability to measure superoxide at low concentrations is of compelling interest for mechanistic studies and quantification of steady state superoxide in natural systems. Foote et al. provides an overview of superoxide sources and sinks as well as discussion of current superoxide detection methods. Foote et al. explains that superoxide “continues to be one of the most difficult of the ROS to quantify accurately in natural waters.” Although its presence can be inferred from numerous lines of evidence, simple unambiguous methods for its determination are lacking.

Currently, the methods that are available for quantitative superoxide concentration determination include direct measurement of the absorbance of superoxide at 240 nm. This method is only practical for pure solutions with concentrations greater than $1 \times 10^{-6}$ M. Nitro blue tetrazolium salt, a chromogenic probe, works at lower $[O_2]$, but probably gives false positive readings with other reducing radicals. Reaction products of superoxide with $^{15}$N-labeled NO(g) rearrange to form nitrate upon protonation. Isotope mass spectrometry is used to follow $^{15}$N in the products to determine the environmental
Figure 3. Absorption spectra of superoxide as a function of irradiation time. Spectra were obtained at time 0, 1, 2, and 3 minutes. Spectra were referenced to non-photolyzed Juice prior to photolysis.
Figure 2. The superoxide production and calibration system ("Juicer"). All tubing was 1/16" ID Teflon tubing, (A). An Osram XBO 75W 2 xenon arc bulb (B) in an Oriel, model 60000 lamp housing (C) was used as the UV light source. The light was directed through a 10 cm quartz cell (D) filled with an alkaline solution of 2-propanol (0.5 M), benzophenone (0.63 μM). A Fluid Metering, Inc. Micro π-petter pump (E) was used to circulate the solution (20 mL/min) through a Hellma 10.0 mm quartz flow cell (F). The absorbance was measured at 240 nm using an Ocean Optics S2000-TR temperature-regulated fiber optic spectrophotometer (G) with an Analytical Instrument Systems model DI 1000 broadband light source (H) both controlled by to a Dell Latitude CPt Computer (I) using an Ocean Optics SAD500 Serial A/D (J).
Figure 4. Flow Injection Analysis System. A Hamamatsu HC124-06 PMT was used to quantify the light flux. The PMT signal was recorded using a DGH 1411 A/D converter. Data acquisition and valve sequencing was controlled using National Instruments LabView software.
All solutions were prepared using 18 MΩ Nanopure water and with 15 μM DTPA added to complex superoxide-scavenging metals. DTPA was used because it strongly binds to metals and doesn’t decay in UV light. The MCLA reagent was stored at 0 °C. Solutions of MCLA were bright yellow and were stored below 10 °C to prevent decomposition. Solutions stored at room temperature decomposed producing a clear solution in a few days. The concentration of MCLA solutions were calculated using the Molecular Probes certified value of ε_{430 \text{ nm}} = 9600 M^{-1} cm^{-1}.

Results and Discussion

Quantification of Stock Solutions. The Photolysis of Juice in the Juicer produced superoxide concentrations around 100 μM after 3 minutes (fig. 3). Juice at pHs 9.6, 11.0, 12.0 was photolyzed and superoxide decay was recorded over time by monitoring the absorbance at 240 nm. Total absorbance of the solution at 240 nm at any time, \( A_T \), was a function of superoxide and hydrogen peroxide concentrations (eqn. 19). For all calculations the path length, \( l \), is 1.0 cm. Curved brackets, {}, will be used to signify the total concentration of all protonated and unprotonated species of the same compound.

\[
A_T = (\{O_2\}, \varepsilon_{O_2} + \{H_2O_2\}, \varepsilon_{H_2O_2}^* )l \tag{19}
\]

The symbol \( \varepsilon_{O_2} \) represents the pH dependent molar extinction coefficients for superoxide found in Table II, and \( \varepsilon_{H_2O_2}^* \) is the total hydrogen peroxide molar absorptivity at 240 nm based on the pH dependent speciation of \( H_2O_2 \). \( H_2O_2 \) is a weak acid, (eqn. 20), with a
pKa = 11.65, so that the correction for its absorbance is significant for the pH range (8-12) of our experiments.

\[ \text{H}_2\text{O}_2 \rightarrow \text{HO}_2^- + \text{H}^+ \quad (20) \]

Equation 21 accounts for the dissociation weighted sum of the molar absorptivity for the protonated and unprotonated peroxide species.

\[ \varepsilon_{\text{H}_2\text{O}_2} = \frac{K_a}{(K_a + [\text{H}^+])} \varepsilon_{\text{HO}_2^-} + \frac{[\text{H}^+]}{(K_a + [\text{H}^+])} \varepsilon_{\text{H}_2\text{O}_2} \quad (21) \]

In our system the lamp intensity is large so that the photolytic production of superoxide is much greater than its decay to hydrogen peroxide. Therefore, there is no significant production of hydrogen peroxide during photolysis. Therefore, the absorbance at the end of photolysis provides the total initial superoxide concentration, C₀ (eqn. 22).

\[ C_0 = \frac{A}{\varepsilon_{\text{O}_2^-}} = \{\text{O}_2^-\} \quad (22) \]

The subsequent absorbance at any time t is then due to the superoxide and hydrogen peroxide that are produced by the dissociation equations 5 and 6.

\[ C_0 = \{\text{O}_2^-\}_0 = \{\text{O}_2^-\}_t + 2\{\text{H}_2\text{O}_2\}_t \quad (23) \]

Where the \{H₂O₂\}_t is described by equation 24.

\[ \{\text{H}_2\text{O}_2\}_t = \frac{1}{2} (C_0 - \{\text{O}_2^-\}_t) \quad (24) \]

Substituting equations 22 and 24 into 19 we get equation 25.

\[ A_t = \{\text{O}_2^-\}_t \varepsilon_{\text{O}_2^-} + \frac{1}{2} \left( \frac{A}{\varepsilon_{\text{O}_2^-}} - \{\text{O}_2^-\}_t \right) \varepsilon_{\text{H}_2\text{O}_2} \quad (25) \]

Which can be rearranged to equation 26.
The second term, 
\[ \frac{1}{2} A_o \left( \frac{\varepsilon_{H_2O_2}}{\varepsilon_{O_2}} \right) = \{O_2\}_i \left( \varepsilon_{O_2} - \frac{1}{2} \varepsilon_{H_2O_2} \right) \]  
(26)

accounts for the absorbance of H₂O₂ at infinite time. This correction is small, and if it is ignored equation 26 then resembles Beilski's term for H₂O₂ corrected absorbance of superoxide (eqn. 27).³

\[ A_i = \{O_2\}_i \left( \varepsilon_{O_2} - \frac{1}{2} \varepsilon_{H_2O_2} \right) \]  
(27)

For careful studies the second term should not be ignored. As \( \varepsilon^* \) becomes greater at higher pHs the absorbance correction term becomes more significant (fig. 5). At pH 12, the term can be as much as 5% of the initial absorbance of the photolyzed solution. At longer and longer decay times the term becomes more significant.

The absorbance data were converted to superoxide concentration using the pH and H₂O₂ correction given by equation 26 (fig. 6). Second-order decay rate constants were obtained from the linear fits to plots of the inverse-concentration vs. time (fig. 7). The data exhibited the expected second order decay at all pHs with the pH 11 and 12 experiments having significantly improved signal to noise due to larger absorbance values and slow decay rates. The experimental decay rate constants, adjusted for 2-propanol,²¹ were plotted vs. pH and compared well with the literature decay rate constants (fig. 8). The decay of superoxide is increased by 2-propanol; therefore, the decay rates of superoxide were decreased by a factor of 2.5 in the adjustment.
Figure 5. Relative absorbance correction term and $\varepsilon^*$ as a function of pH. The $\varepsilon^*$ (triangles) is calculated using equation 21 and the $\varepsilon$ values for $\text{H}_2\text{O}_2$, $\text{HO}_2^-$ are found in table II. The relative absorbance correction to solution absorbance at $t = 0$ (squares) term was calculated from $\frac{1}{2} A_0 \left( \frac{\varepsilon_{\text{H}_2\text{O}_2}}{\varepsilon_{\text{O}_2^*}} \right)$. This term will increase linearly as the $[\text{O}_2]$ decreases by half.
Figure 6. Decay of superoxide measured by absorbance at 240 nm in the Juicer. Juice buffered with NaOH to pH 12.0 (triangles), pH 11.0 (squares), and pH 9.6 (circles). Second order decay curves fit to data (solid black curves).
Figure 7. Inverse superoxide concentration vs. time from decay observed by Juicer. Solid gray lines are linear fits to the data.
Figure 8. Second-order decay rate constants of superoxide in Juicer from the measurement of absorbance at 240 nm vs. pH (diamonds), corrected (squares) by a factor of 2.5 due to the known enhancement of superoxide decay by 2-propanol$^{21}$ and plotted against known superoxide decay rate constants$^{3}$ (line).
Since second order kinetics require the exact starting concentration, these determinations were necessarily thorough. From equation 26 we can see that the extra H2O2 correction term becomes more important as the pH increases due to its dependence on $e_{H_2O_2}$. Hydrogen peroxide is an important chromophore so repeated photolysis will not yield quantifiable amounts of superoxide by absorbance at 240 nm, since equation 26 assumes that the concentration of superoxide is initially zero.

Superoxide decay constants measured by the Juicer are in excellent agreement with published decay constants.\(^3\) We can therefore assert with confidence that the system produces quantitative superoxide stocks (5 -100 \(\mu\)M), which are stable at pH 12. These stocks are readily quantified at any time by absorbance.

**Calibration of MCLA.** Standard additions of superoxide from stock solutions were added to a pH 12.0 buffered sample for low level superoxide analysis. The sample was mixed with MCLA in the FIA flow cell (fig. 9) and a standard calibration curve was calculated. The response was linear ($R^2 = 0.999$) and had a detection limit (3 \(\sigma\)) of 0.074 nM superoxide (fig. 10).

Since the additions of superoxide could not be measured instantaneously in the FIA system, concentrations of superoxide were corrected for the expected decay to peroxide from the time of addition. However, since the standard additions were made at pH 12, the decay of superoxide over several minutes was small. The PMT signal response to each addition was consistently 375 - 750 mV per nM superoxide added. MCLA chemiluminescence was 10,000 times less for comparable additions of H2O2 and completely unresponsive to 2-propanol and benzophenone additions.
Figure 9. FIA-MCLA-CL response peaks from the superoxide calibration curve.
Figure 10. Calibration curve for the FIA-MCLA system. Known additions of \([O_2^-]\) from stock solution into a pH 10.5 NaOH solution. The solution was injected into the sample line of the flow injection system with MCLA (22.7 \(\mu\)M) in pH 4.94 sodium acetate buffer in the reagent line. The waste from the injection was at pH 5.13.
Superoxide Decay Studies of MCLA. Superoxide decay rates were studied using our FIA-MCLA system to further evaluate the analytical system. A series of solutions were buffered with Tris from pH 8.0 to 9.1 with superoxide concentrations (100 nM) prepared from superoxide stock solutions. These solutions were added individually to the FIA-MCLA system and the decay of superoxide was measured over time for each sample (fig. 11). The response of MCLA to superoxide was consistent over the range of sample pHs. Since the MCLA pH was kept buffered at 5.1, the pH of the reaction was constant at 5.1. The similarity of second-order decay rates based on MCLA CL confirms that superoxide is being measured by the MCLA-FIA system (fig. 12).

Natural Samples. A pond water sample from Great Pond (Belgrade Lakes, ME) with 15 µM DTPA added to complex superoxide scavenging metals was irradiated with an Osram XBO 75 2 xenon arc lamp to investigate the photochemical production of superoxide. A sample was drawn directly from the irradiation vessel and superoxide production and decay was observed from the FIA-MCLA system. No superoxide was present before photolysis. Upon photolysis, superoxide rapidly formed to steady concentrations (1.2 nM), which rapidly decayed in the dark (fig. 13). Qualitative attempts to fit the data were compared to the plot, but need refinement using replicates of many natural water types.
Figure 11. Decay of superoxide determined by FIA-MCLA signal at various pHs. Quantitative superoxide additions from stock to sample solutions buffered with Tris to pH 9.1 (diamonds), 8.6 (circles), and 8.0 (squares). The reagent MCLA (3.75 μM) was dissolved in acetate buffer (0.1 M) at pH 5.1.
Figure 12. Second-order decay of superoxide as measured by MCLA (triangles) and as measured by Juicer (squares)
Figure 13. Photolysis of a natural pond water sample.
Conclusions

In summary, stock solutions of 100 μM superoxide were produced and quantified simultaneously in a circulating flow system. Quantified additions of the stock solution were used to calibrate the CL response of FIA-MCLA. Juice absorbance and FIA-MCLA-CL demonstrated pH-dependent decay rates of superoxide similar to published rates. Detection limits were observed at 0.074 nM superoxide.

Future Work

The entire FIA system needs to be optimized in terms of the buffer pH of MCLA, concentration of MCLA, and flow rates. These terms can affect the reaction rates of CL light production, so optimization is needed to exploit the full detection potential of the system. For natural samples, more work needs to be done, including quantitative superoxide additions to determine matrix and interference effects in these samples. Photochemical production of superoxide in the Juicer should be modeled, through rate studies, to give the optimum photolysis conditions for clean superoxide production.
References


