A new chemiluminescence probe for singlet oxygen based on tetrathiafulvalene-anthracene dyad capable of performing detection in water/alcohol solution

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Abstract

A new tetrathiafulvalene-anthracene dyad 1 with two “tetraethylene glycol” units was synthesized and characterized. Strong chemiluminescence was observed upon reaction of dyad 1 with singlet oxygen (1O2), and this reaction shows fairly good selectivity toward 1O2 over other reactive oxygen species. Due to the introduction of two hydrophilic “tetraethylene glycol” units, the detection of 1O2 with dyad 1 can be performed in alcohol/water solution, which is relatively a mild medium when compared with water/tetrahydrofuran solution required by other tetrathiafulvalene-anthracene dyads. Dyad 1 may have a wider use for detection of 1O2 in biological systems.

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1. Introduction

It is well known that 1O2 is an important oxidation species in biological processes [1,2]. Several enzyme systems have been identified as biochemical sources of 1O2. They include lipooxygenase, peroxidase and eosinophil peroxidase [3–5]. Evidence has accumulated indicating that 1O2 is implicated in the genotoxic effect of the ultra violet A (320–400 nm) component of solar radiation and that it likely plays an important role in the cell signaling cascade and in the induction of gene expression [6,7]. To understand the role of 1O2 involved in these processes in depth, invention of reliable detection methods for 1O2 is essential. However, development of highly selective and sensitive spectroscopic probe for 1O2 still remains to be challenging because of the following aspects: (1) 1O2 shows properties similar to those of other reactive oxygen species (ROS) and it is difficult to distinguish from other ROS; (2) the production yield of 1O2 is low and also its lifetime is short (2–4 μs) in aqueous environments [8].

A number of spectroscopic methods for the detection of 1O2 have been developed. Direct detection of 1O2 by monitoring the emission at 1270 nm is a specific and noninvasive method, but the low emission efficiency for 1O2 sometimes prohibits the practical application in biological systems [9]. Absorbance-based probes have been also developed by making use of the rather specific reactions of 1O2 with these probes [10,11], such as 9,10-diphenylanthracene [12], 2,5-dimethylfuran [13], and furfuryl alcohol [14]; the analysis of 1O2 is based on the corresponding absorption spectral changes of these probes after reaction with 1O2. However, absorbance-based detection is inherently less sensitive than luminescence detection. More sensitive fluorescent probes for 1O2 have been also reported. For example, Nagano and co-workers have recently described a sensitive fluorescent 1O2 probe based on the fluorescein derivative 9-[2-(3-carboxy-9,10-dimethyl)anthryl]-6-hydroxy-3H-xanthen-3-one, which shows large fluorescence enhancement after specific reaction with 1O2 [15,16]. In addition, Yuan and co-workers have reported a new europium chelate-based phosphorescence probe for 1O2 with a detection limit of 2.8 nM [17,18].

Among the most sensitive 1O2 probes are those in which detection is based on chemiluminescence (CL). For chemilu-
Synthesis and chemiluminescence properties of dyad 1 for $^1$O$_2$ assay.

2. Experimental

2.1. Reagents and materials

Triisopropylphosphite and CsOH-H$_2$O were purchased from Acros, while lactoperoxidase ($A_{412\text{ nm}}/A_{280\text{ nm}} = 0.76$; specific activity = 111 units mg$^{-1}$ of protein) was from Sigma. Hydrogen peroxide, sodium hypochlorite and deuterium oxide were obtained from Beijing Chemical Company. Prior to use, hydrogen peroxide was diluted immediately from a stabilized 30% solution, and was assayed by using 43.6 M$^{-1}$ cm$^{-1}$ as the molar absorptivity at 240 nm [29]. Hypochlorous acid was prepared by distillation from the 5% commercial sodium hypochlorite solution and stored, for periods less than one week, at 4°C as a 300 mM solution with a pH of 11 adjusted by the addition of sodium hydroxide. Before use, sodium hypochlorite was assayed using a molar absorptivity of 391 M$^{-1}$ cm$^{-1}$ at 292 nm [30]. The stock solution of dyad 1 200 µM was prepared in methanol. Deuterium oxide (99.8% purity) was used without further purification. All other chemicals were local products of analytical grade. Deionized and distilled water was used throughout.

Compound 2 was synthesized according to the procedures described previously [31]. The synthesis of compound 3 was performed by following the procedures reported in ref. [32], in which the use of the cyanoethyl group as a thiol protecting group was detailed.
2.2. Instruments

$^1$H NMR spectra and $^{13}$C NMR were recorded on a BRUCK 400 MHz. Mass spectra data were determined with APEX II (Bruker Inc.).

Lumat LB 9507 (EG & G BERTHOLD, Bad Wildbad, Germany) was used for CL measurements. This apparatus equipped with a variable automatic volume injector has a function of monitoring kinetic behavior of light emission; the emitted light is measured with a high sensitivity, low noise photo multiplier. Its spectral sensitivity covers a range of 390–620 nm. CL and fluorescence spectra were recorded with a Hitachi F-2500 spectrofluorimeter; for the measurement of CL spectrum, the excitation light source was switched off. A model 25 pH-meter was used for pH measurements.

2.3. Synthesis of dyad 1

A solution of compound 2 [31] (0.25 g, 0.65 mmol) and compound 3 [32] (0.50 g, 1.74 mmol) in triisopropylphosphite (5 mL) was heated to 120 °C under N₂ and stirred at this temperature for 3 h. After removing triisopropyl phosphate under reduced pressure, the resulting crude product was purified by column chromatography, giving compound 4 (0.20 g, 50% yield) as yellow oil. $^1$H NMR (400 MHz, CDCl₃): 8.30 (d, 2H), 8.26 (s, 1H), 8.01 (d, 2H), 7.49 (m, 4H), 6.53 (s, 1H), 4.42 (t, 2H), 3.38 (t, 2H), 3.07 (t, 4H), 2.71 (t, 4H). $^{13}$C NMR (100 MHz, CDCl₃): 150.2, 132.3, 128.5, 128.0, 126.6, 125.4, 123.0, 122.7, 122.1, 117.6, 117.5, 106.0, 73.3, 36.1, 31.2, 18.8. Anal. cacld. for C₃₈H₄₈O₉S₇: 872.1330 (HR-MS). The synthesis of dyad 4 started from the coupling of compound 2 [31] and leading to 4 in 50% yield (Scheme 1). Removal of the two cyanoethyl groups in compound 4 in the presence of CsOH and further reaction with 1-hydroxy-3,6,9-trioxaundexyl p-toluenesulfonate [33] led to 1 in 39% yield. Their structures were confirmed by spectroscopic methods.

3.2. CL reaction with $^{1}$O₂ and other ROS

As expected, strong chemiluminescence was observed for the solution of dyad 1 upon reaction with $^{1}$O₂. Fig. 1 shows the CL spectrum together with the corresponding fluorescence spectrum of dyad 1 upon reaction with $^{1}$O₂. It can be concluded that the CL generated from the reaction of dyad 1 and $^{1}$O₂ is from the excited state of the anthracene unit of dyad 1 as discussed for other tetrathiafulvalene-anthracene dyads reported previously [26,27]. Dyad 1 was also allowed to react with other ROS to examine its selectivity toward $^{1}$O₂. The results are summarized in Table 1.

![Fig. 1. CL spectrum (—) of 1 and its fluorescence spectrum (...) after reaction with $^{1}$O₂. The concentrations of reactants were 200 μM of 1, 10 mM of $\text{H}_2\text{O}_2$ and 30 mM of NaOCl. After oxidation and an appropriate dilution the reaction solution was then used to measure the fluorescence spectrum.](image-url)
Table 1
Comparison of relative CL intensities from the reaction of dyad 1 with different ROS

<table>
<thead>
<tr>
<th>Reagent blank</th>
<th>( ^{1}\text{O}_2 )</th>
<th>( \text{H}_2\text{O}_2 )</th>
<th>OH(^{-})</th>
<th>( \text{O}_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7</td>
<td>0.007 ± 0.001</td>
<td>1.000 ± 0.040</td>
<td>0.010 ± 0.001</td>
<td>0.009 ± 0.001</td>
</tr>
</tbody>
</table>

\(^{a}\) The CL intensity (4.7 \( \times \) \( 10^{3} \) RLU) from the reaction of dyad 1 with \( ^{1}\text{O}_2 \) at pH 7 was defined as 1.0. CL reaction was initiated by injecting appropriate amount of ROS into 50 mM sodium phosphate buffer of pH 7 containing 20 \( \mu \)M of probe and 20\% (v/v) MeOH as a cosolvent at 25 °C. The data were expressed as the mean of three determinations ± standard deviation.

\(^{b}\) 1.0 mM \( \text{H}_2\text{O}_2 \) + 10 mM NaOCl.

\(^{c}\) 1.0 mM \( \text{H}_2\text{O}_2 \).

\(^{d}\) 1.0 mM \( \text{H}_2\text{O}_2 \) + 0.1 mM ferrous ammonium sulfate.

\(^{e}\) 0.1 mL of superoxide solution (1.0 mg KO\(_2\)/mL DMSO).

in Table 1. Clearly, strong chemiluminescence was only detected in the case of \( ^{1}\text{O}_2 \) compared with other ROS (see Table 1). Thus, dyad 1 as a CL probe displays high selectivity toward \( ^{1}\text{O}_2 \). But, it should be noted that the corresponding tetraphiafulvalene-anthracene dyads as CL probes for \( ^{1}\text{O}_2 \) without “tetraethylene glycol” units show even higher selectivity toward \( ^{1}\text{O}_2 \) [27].

It is known that the reaction of \( \text{H}_2\text{O}_2 \) with NaOCl can generate \( ^{1}\text{O}_2 \) efficiently. For this reason the reaction of \( \text{H}_2\text{O}_2 \) with NaOCl at pH 7.0 was employed to calibrate the concentration of \( ^{1}\text{O}_2 \). Similar to other tetraphiafulvalene-anthracene dyads [27], the CL rate observed for the solution of dyad 1 in the presence of \( \text{H}_2\text{O}_2 \)/NaOCl is very fast, and a measuring time of 5 s for recording the CL signal intensity can be used. The CL intensities of dyad 1 after reaction with \( ^{1}\text{O}_2 \) over the concentration range of 0.0025–4.0 mM were measured. Over this whole concentration range, the plot of the CL intensity versus the concentration of \( ^{1}\text{O}_2 \) showed poor linear relation. This is likely due to the fact that different species are involved in the rate-limiting step at different concentrations of \( ^{1}\text{O}_2 \). But, relatively good linear relations were obtained in two separate concentration ranges (see Fig. 2):

\[ I_{\text{CL}} = (3.25 \pm 0.11) \times 10^{4} \cdot C^{-(11 \pm 7)} \quad (n = 6, \gamma = 0.998) \]

in the concentration range of 0.0025–0.01 mM, and

\[ I_{\text{CL}} = (3.33 \pm 0.13) \times 10^{3} \cdot C + (662 \pm 229) \quad (n = 7, \gamma = 0.996) \]

in the concentration range of 0.04–4.0 mM. Based on the linear relation at low concentration range, the detection limit for \( ^{1}\text{O}_2 \) was estimated to be 1.0 \( \mu \)M based on 11 blank determinations (\( k = 3 \)). It is noteworthy that this CL probe shows low sensitivity compared to the previous TTF-anthracene dyads [26,27]. The reason may be that the CL intensities of this probe were measured in the mixture of methanol and water, and according to the known fact [34], solvents with hydroxyl groups would shorten the lifetime of \( ^{1}\text{O}_2 \). As a result, the reaction of the anthracene unit with \( ^{1}\text{O}_2 \) may be affected, and a low sensitivity towards \( ^{1}\text{O}_2 \) may be resulted. With the determination of 0.007 mM of \( ^{1}\text{O}_2 \) as an example, reproducibility test (\( n = 7 \)) showed that the relative standard deviation of CL intensity was 4\%, indicating an acceptable level of precision.

Our recent studies showed that the effect of common ions such as K\(^{+}\), Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), Zn\(^{2+}\), Al\(^{3+}\), Cl\(^{-}\), HCO\(_3\)\(^{-}\), NO\(_3\)\(^{-}\) and SO\(_4\)\(^{2-}\) on the CL of the TTF-anthracene dyad upon reaction with \( ^{1}\text{O}_2 \) was not significant [21]. According to the mechanism proposed for the reaction of TTF-anthracene dyad with \( ^{1}\text{O}_2 \) [26,27], the neutral TTF unit in the dyad plays an important role for the production of strong CL. Accordingly, the oxidants such as Fe\(^{3+}\) and Cu\(^{2+}\), which do not react with the anthracene unit but they can oxidize the TTF unit leading to the corresponding cation species, may interfere with the determination of \( ^{1}\text{O}_2 \). But, the presence of low concentrations of Fe\(^{3+}\) and Cu\(^{2+}\) produced no obvious influence on the CL as indicated by our recent report [21].

It is reported that the system of lactoperoxidase/\( \text{H}_2\text{O}_2 \)/Br\(^{-}\) can produce \( ^{1}\text{O}_2 \) [35,36]. Dyad 1 was used to detect \( ^{1}\text{O}_2 \) from

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Fig. 2. The dependence of CL intensity of dyad 1 (20 \( \mu \)M) on \( ^{1}\text{O}_2 \) generated from the reaction of 10 mM of NaOCl with a series of \( \text{H}_2\text{O}_2 \) concentrations: 0.0025–0.01 mM and 0.04–4.0 mM. The calibration curve was derived from the integrated CL signal of the \( \text{H}_2\text{O}_2 \)/NaOCl/dyad 1 over a 5 s period minus that of the corresponding reagent blank (NaOCl/dyad 1) without \( \text{H}_2\text{O}_2 \). The reaction was carried out in 50 mM sodium phosphate buffer of pH 7.0 containing 20 \( \mu \)M of probe and 20\% (v/v) MeOH as a cosolvent.
such a system to show its potential to be used for biological systems. After addition of dyad 1 to the lactoperoxidase/H$_2$O$_2$/Br$^-$ system in water/methanol (80:20, v/v) solution, comparably strong chemiluminescence was detected (Table 2). If D$_2$O was introduced (system 2, Table 2), large enhancement (40%) of the CL intensity of system 1 was observed. It was reported that the lifetime of 1O$_2$ in D$_2$O was longer than that in H$_2$O [8]. As a result, more 1O$_2$ would be trapped by the anthracene unit after introduction of D$_2$O in the system, leading to the increase of the CL intensity. Therefore, this result was in agreement with the fact that 1O$_2$ was generated from the lactoperoxidase/H$_2$O$_2$/Br$^-$ system [35,36]. Further evidence for the involvement of 1O$_2$ was from the significant CL quenching (92%) by addition of azide (system 4), which was reported to be an efficient quencher was from the significant CL quenching (92%) by addition of azide (system 4), which was reported to be an efficient quencher [21,26,27,37]. It should be pointed out that azide also not only a scavenger of 1O$_2$ but also an inhibitor of peroxi-
dase [38]. These results clearly corroborate the production of 1O$_2$ from the system of lactoperoxidase/H$_2$O$_2$/Br$^-$ - quantita-
tively, a 1O$_2$ yield of 0.14 mM was thus obtained for the system 1 over a 60 s reaction period based on the above calibration curve (in the concentration range of 0.04–4.0 mM) constructed with the H$_2$O$_2$/NaOCl/dyad 1 system, after subtracting the back-
ground signal (system 3, Table 2). Similarly, the measurement of 1O$_2$ with dyad 1 can be also carried out in water/ethanol solution. Compared with other tetraphiafulvene-anthracene dyads, which have to be used in water/THF solution for the detection of 1O$_2$ [27], the application of dyad 1 as 1O$_2$ probe in water/alcohol solution is one step closer for the practical use for detection of 1O$_2$ in biological systems.

4. Conclusion

A new tetraphiafulvene-anthracene dyad 1 was synthesized and characterized. Similar to other tetraphiafulvene-anthracene dyads reported previously [21,26,27], strong chemiluminescence was observed upon reaction of dyad 1 with 1O$_2$, and this reaction shows fairly good selectivity toward 1O$_2$ over other ROS. Due to the introduction of hydrophilic “tetraethylene gly-
col” units, dyad 1 can be easily dissolved in methanol or ethanol, thus the detection of 1O$_2$ with dyad 1 can be performed in alcohol/water solution. Further researches include design and studies of the tetraphiafulvene-anthracene dyads that show good sol-

Table 2

<table>
<thead>
<tr>
<th>Relative CL intensities from different reaction systems$^a$</th>
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<tbody>
<tr>
<td>1. Dyad 1 + H$_2$O$_2$ + Br$^-$ + lactoperoxidase + buffer</td>
</tr>
<tr>
<td>2. System 1 containing 50% D$_2$O (v/v)</td>
</tr>
<tr>
<td>3. Dyad 1 + H$_2$O$_2$ + lactoperoxidase + buffer</td>
</tr>
<tr>
<td>4. System 1 containing 5 mM Na$_3$</td>
</tr>
<tr>
<td>5. System 3 containing 5 mM Na$_3$</td>
</tr>
</tbody>
</table>

$^a$ The CL intensity (1789 RLU) from the reaction of dyad 1 with 1O$_2$ produced by lactoperoxidase at pH 4.5 was defined as 1.0. CL reaction was initiated by injecting 25 μL of 40 mM H$_2$O$_2$ (final concentration 1 mM) into 0.1 M sodium acetate buffer (pH 4.5), containing 20 μM of dyad 1, 0.1 mg/mL lactoperoxidase, 20 mM Br$^-$ and 20% (v/v) MeOH as a cosolvent at 25°C; the CL intensity was measured over a 60 s period. The data were expressed as the mean of three determinations ± standard deviation.

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