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 (1O\textsubscript{2}), and this reaction shows fairly good selectivity toward 1O\textsubscript{2} over other reactive oxygen species. Due to the introduction of two hydrophilic “tetraethylene glycol” units, the detection of 1O\textsubscript{2} 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 1O\textsubscript{2} in biological systems.

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1. Introduction

It is well known that 1O\textsubscript{2} is an important oxidation species in biological processes [1,2]. Several enzyme systems have been identified as biochemical sources of 1O\textsubscript{2}. They include lipoxygenase, peroxidase and eosinophil peroxidase [3–5]. Evidence has accumulated indicating that 1O\textsubscript{2} 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 1O\textsubscript{2} involved in these processes in depth, invention of reliable detection methods for 1O\textsubscript{2} is essential. However, development of highly selective and sensitive spectroscopic probe for 1O\textsubscript{2} still remains to be challenging because of the following aspects: (1) 1O\textsubscript{2} 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 1O\textsubscript{2} is low and also its lifetime is short (2–4 \textmu s) in aqueous environments [8].

A number of spectroscopic methods for the detection of 1O\textsubscript{2} have been developed. Direct detection of 1O\textsubscript{2} by monitoring the emission at 1270 nm is a specific and noninvasive method, but the low emission efficiency for 1O\textsubscript{2} 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 1O\textsubscript{2} with these probes [10,11], such as 9,10-diphenylanthracene [12], 2,5-dimethylfuran [13], and furfuryl alcohol [14]; the analysis of 1O\textsubscript{2} is based on the corresponding absorption spectral changes of these probes after reaction with 1O\textsubscript{2}. However, absorbance-based detection is inherently less sensitive than luminescence detection. More sensitive fluorescent probes for 1O\textsubscript{2} have been also reported. For example, Nagano and co-workers have recently described a sensitive fluorescent 1O\textsubscript{2} 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 1O\textsubscript{2} [15,16]. In addition, Yuan and co-workers have reported a new europium chelate-based phosphorescence probe for 1O\textsubscript{2} with a detection limit of 2.8 nM [17,18].

Among the most sensitive 1O\textsubscript{2} probes are those in which detection is based on chemiluminescence (CL). For chemilu-
Scheme 1. Synthesis of dyad 1: (i) (i-PrO)₃P, 120 °C, 3 h; (ii) CsOH·H₂O, 1-hydroxy-3,6,9-trioxaundexyl-p-toluenesulfonate.

minescent \(^1\)O₂ probes, no light source is needed. As a result, they can be applied to eliminating background fluorescence and various light scattering to improve signal-noise ratio. Moreover, CL measurements do not require sophisticated equipment. The Cypridina luciferin analogues are widely studied as the CL probes for \(^1\)O₂ [19,20], and a detection limit for \(^1\)O₂ has been reported to be 9.4 nM [21]; but these CL probes show low selectivity and high CL background. CL probes for \(^1\)O₂ based the chemistry of 1,2-dioxetanes have been reported [22,23]. For instance, McNeill and coworkers have investigated stable dioxetane precursors as selective probes for \(^1\)O₂ which show high sensitivity as indicated by the fact that \(5 \times 10^{-13}\) M of \(^1\)O₂ can be accurately measured with these probes [24,25]. We have recently reported two highly selective and sensitive (with detection limits of about 80 nM for \(^1\)O₂) chemiluminescence probes based on tetrathiafulvalene-anthracene dyads for the detection of \(^1\)O₂ [26,27]. But, due to their low water solubility, the detection of \(^1\)O₂ has to be carried out in a mixture solvent of tetrahydrofuran (THF) and H₂O. Obviously, it would be advantageous if the detection could be performed in aqueous solution. However, only limited water-soluble CL probes are available so far for \(^1\)O₂ assay, particularly for \(^1\)O₂ selective detection [25,28]. For that purpose, we designed and synthesized a new tetrathiafulvalene-anthracene dyad 1 (Scheme 1), into which two “tetraethylene glycol” units are incorporated. The results show that dyad 1 still has low solubility in water, but it can be easily dissolved in ethanol and methanol, which may permit the detection of \(^1\)O₂ in a relatively mild medium such as alcohol/water solution. In this paper, we will report the synthesis and chemiluminescence properties of dyad 1 for \(^1\)O₂ assay.

2. Experimental

2.1. Reagents and materials

Triisopropylphosphite and CsOH·H₂O were purchased from Acros, while lactoperoxidase (\(A_{412\text{ nm}}/A_{280\text{ nm}} = 0.76; \text{specific activity} = 111 \text{ units mg}^{-1} \text{ 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 BRUCK400 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 N$_2$ and stirred at this temperature for 3 h. After removing triisopropyl phosphite 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, CDCl$_3$): 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, CDCl$_3$): 150.0, 132.2, 128.5, 128.0, 126.4, 125.5, 125.4, 122.7, 122.1, 117.6, 117.5, 106.0, 73.3, 36.1, 31.2, 18.8. Anal. cacld. for C$_{38}$H$_{48}$O$_9$S$_7$: 872.1338; found: 872.1330 (HR-MS).

To a solution of 4 (0.21 g, 0.33 mmol) in anhydrous degassed THF (20 mL) was added a solution of CsOH-H$_2$O (0.13 g, 0.79 mmol) in anhydrous degassed MeOH (5 mL) over a period of 10 min. The mixture was stirred for an additional 30 min whereupon a solution of degassed 1-hydroxy-3,6,9-trioxaundexyl p-toluenesulfonate [33] (0.342 g, 0.983 mmol) in anhydrous degassed THF (10 mL) was added. The solution was stirred overnight. After separation by column chromatography, compound 1 was obtained in 39% yield (0.11 g). The product is readily soluble in methanol, ethanol, and THF. $^1$H NMR (400 MHz, CDC$_3$): 8.33 (d, 2H), 8.24 (s, 1H), 8.00 (d, 2H), 7.48 (m, 4H), 6.50 (s, 1H), 4.40 (t, 2H), 3.71-3.57 (m, 28H), 3.38 (t, 2H), 3.01 (t, 4H), 1.87 (br, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): 150.2, 132.3, 128.5, 128.0, 127.9, 126.4, 125.5, 125.4, 154.5, 123.2, 122.6, 122.1, 114.4, 108.5, 73.4, 72.5, 70.4, 61.7, 36.0, 35.4, 29.7. Anal. cacld. for C$_{38}$H$_{48}$O$_9$S$_7$: 872.1338; found: 872.1330 (HR-MS).

2.4. CL reaction and detection

All experiments were made at 25°C in 50 mM sodium phosphate buffer (pH 7) containing 20% (v/v) methanol as a cosolvent unless otherwise noted. Typically, a 1 mL portion of the phosphate buffer containing 20 μM of the probe 1 and an appropriate concentration of the reactant (e.g., 1 mM hydrogen peroxide or other ROS) was placed in a test tube in the CL detector. The reaction was initiated by rapid automatic injection of 0.1 mL sodium hypochlorite, and CL was measured as the integral of the CL intensity in RLU (relative light units) over the total reaction period (typically 5 s) with Lumat LB 9507 luminometer. Unless otherwise stated, each data was expressed as the mean of three determinations with a relative error of less than ±5%.

ROS production and $^1$O$_2$ detection were carried out based on previously reported procedures [26,27]. Quantitative measurements of $^1$O$_2$ production were made according to the Kanofsky’s method [3–5]. As an example, the production of $^1$O$_2$ from the system of lactoperoxidase/H$_2$O$_2$/Br$^-$ was determined with dyad 1 as a probe based on the calibration curve obtained from the H$_2$O$_2$/NaOCl/I reaction in phosphate buffers with 20 μM of 1, 10 mM of NaOCl and a series of H$_2$O$_2$ concentrations of 1.0 mM or less.

3. Results and discussion

3.1. Synthesis and characterization

The synthesis of dyad 1 started from the coupling of 2 [31] and 3 [32] 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 $^1$O$_2$ and other ROS

As expected, strong chemiluminescence was observed for the solution of dyad 1 upon reaction with $^1$O$_2$. Fig. 1 shows the CL spectrum together with the corresponding fluorescence spectrum of dyad 1 upon reaction with $^1$O$_2$. It can be concluded that the CL generated from the reaction of dyad 1 and $^1$O$_2$ is from the excited state of the anthracene unit of dyad 1 as discussed for other tetrahydrofulvalene-anthracene dyads reported previously [26,27]. Dyad 1 was also allowed to react with other ROS to examine its selectivity toward $^1$O$_2$. The results are summarized...
Table 1
Comparison of relative CL intensities from the reaction of dyad 1 with different ROS

<table>
<thead>
<tr>
<th>pH 7</th>
<th>Reagent blank</th>
<th>O₂ b</th>
<th>H₂O₂ c</th>
<th>OH⁻ d</th>
<th>O₂ e</th>
</tr>
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<tbody>
<tr>
<td></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>
<td>0.009 ± 0.001</td>
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</table>

a The CL intensity (4.7 × 10³ RLU) from the reaction of dyad 1 with O₂ 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 µM 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 H₂O₂ + 10 mM NaOCl.
c 1.0 mM H₂O₂.
d 1.0 mM H₂O₂ + 0.1 mM ferrous ammonium sulfate.
e 0.1 mL of superoxide solution (1.0 mg KO₂/mL DMSO).

in Table 1. Clearly, strong chemiluminescence was only detected in the case of O₂ compared with other ROS (see Table 1). Thus, dyad 1 as a CL probe displays high selectivity toward O₂. But, it should be noted that the corresponding tetrathiafulvalene-anthracene dyads as CL probes for O₂ without “tetraethylene glycol” units show even higher selectivity toward O₂ [27].

It is known that the reaction of H₂O₂ with NaOCl can generate O₂ efficiently. For this reason the reaction of H₂O₂ with NaOCl at pH 7.0 was employed to calibrate the concentration of O₂. Similar to other tetrathiafulvalene-anthracene dyads [27], the CL rate observed for the solution of dyad 1 in the presence of H₂O₂/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 O₂ 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 O₂ 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 O₂. But, relatively good linear relations were obtained in two separate concentration ranges (see Fig. 2): \( I_{CL} = (3.25 ± 0.11) \times 10^4 \cdot C^{-(11 ± 7)} \) (\( n = 6, \gamma = 0.998 \)) in the concentration range of 0.0025–0.01 mM, and \( I_{CL} = (3.33 ± 0.13) \times 10^3 \cdot C + (662 ± 229) \) (\( n = 7, \gamma = 0.996 \)) in the concentration range of 0.04–4.0 mM. Based on the linear relation at low concentration range, the detection limit for O₂ was estimated to be 1.0 µM 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 O₂. As a result, the reaction of the anthracene unit with O₂ may be affected, and a low sensitivity towards O₂ may be resulted. With the determination of 0.007 mM of O₂ 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⁺, Ca²⁺, Mg²⁺, Mn²⁺, Ni²⁺, Zn²⁺, Al³⁺, Cl⁻, HCO₃⁻, NO₃⁻ and SO₄²⁻ on the CL of the TTF-anthracene dyad upon reaction with O₂ was not significant [21]. According to the mechanism proposed for the reaction of TTF-anthracene dyad with O₂ [26,27], the neutral TTF unit in the dyad plays an important role for the production of strong CL. Accordingly, the oxidants such as Fe³⁺ and Cu²⁺, 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 O₂. But, the presence of low concentrations of Fe³⁺ and Cu²⁺ produced no obvious influence on the CL as indicated by our recent report [21].

It is reported that the system of lactoperoxidase/H₂O₂/Br⁻ can produce O₂ [35,36]. Dyad 1 was used to detect O₂ from
such a system to show its potential to be used for biological systems. After addition of dyad 1 to the lactoperoxidase/H2O2/Br− system in water/methanol (80:20, v/v) solution, comparably strong chemiluminescence was detected (Table 2). If D2O 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 1O2 in D2O was longer than that in H2O [8]. As a result, more 1O2 would be trapped by the anthracene unit after introduction of D2O in the system, leading to the increase of the CL intensity. Therefore, this result was in agreement with the fact that 1O2 was generated from the lactoperoxidase/H2O2/Br− system [35,36]. Further evidence for the involvement of 1O2 was from the significant CL quenching (92%) by addition of azide (system 4), which was reported to be an efficient quencher for 1O2 [21,26,27,37]. It should be pointed out that azide also quenched the CL of control sample (system 5), since azide is not only a scavenger of 1O2 but also an inhibitor of peroxidase [38]. These results clearly corroborate the production of 1O2 from the system of lactoperoxidase/H2O2/Br− system [5]. Quanti- tatively, a 1O2 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 H2O2/NaOCl/dyad 1 system, after subtracting the background signal (system 3, Table 2). Similarly, the measurement of 1O2 with dyad 1 can be also carried out in water/ethanol solution. Compared with other tetraethylfulvene-anthracene dyads, which have to be used in water/THF solution for the detection of 1O2 [27], the application of dyad 1 as 1O2 probe in water/alcohol solution is one step closer for the practical use for detection of 1O2 in biological systems.

4. Conclusion

A new tetraethylfulvene-anthracene dyad 1 was synthesized and characterized. Similar to other tetraethylfulvene-anthracene dyads reported previously [21,26,27], strong chemiluminescence was observed upon reaction of dyad 1 with 1O2, and this reaction shows fairly good selectivity toward 1O2 over other ROS. Due to the introduction of hydrophilic “tetaethylene glycol” units, dyad 1 can be easily dissolved in methanol or ethanol, thus the detection of 1O2 with dyad 1 can be performed in alcohol/water solution. Further researches include design and studies of the tetraethylfulvene-anthracene dyads that show good usability in fully aqueous solutions, thereby making them to be more useful for biological studies.

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