A chemiluminescent probe for cellular peroxynitrite using a self-immolative oxidative decarbonylation reaction†

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Peroxynitrite (ONOO⁻) is a highly reactive oxygen species which has been recognized as an endogenous mediator of physiological activities like the immune response as well as a damaging agent of oxidative stress under pathological conditions. While its biological importance is becoming clearer, many of the details of its production and mechanism of action remain elusive due to the lack of available selective and sensitive detection methods. Herein, we report the development, characterization, and biological applications of a reaction-based chemiluminescent probe for ONOO⁻ detection, termed as PNCL. PNCL reacts with ONOO⁻ via an isatin moiety through an oxidative decarbonylation reaction to initiate light emission that can be observed instantly with high selectivity against other reactive sulphur, oxygen, and nitrogen species. Detailed studies were performed to study the reaction between isatin and ONOO⁻, which confirm selectivity for ONOO⁻ over NO₂⁻. PNCL has been applied for ONOO⁻ detection in aqueous solution and live cells. Moreover, PNCL can be employed to detect cellular ONOO⁻ generated in macrophages stimulated to mount an immune response with lipopolysaccharide (LPS). The sensitivity granted by chemiluminescence detection together with the specificity of the oxidative decarbonylation reaction provides a useful tool to explore ONOO⁻ chemistry and biology.

Introduction

Peroxynitrite (ONOO⁻) is a highly reactive oxygen species that can be formed biologically from a diffusion-controlled reaction between superoxide (O₂⁻) and nitric oxide (NO).1 In biological systems, ONOO⁻ has long been known as a deleterious species due to its oxidative damage to lipids, proteins, and nucleic acids.2 Abnormal regulation of ONOO⁻ in living systems is associated with various diseases such as ischemia-reperfusion,1 diabetes,3 cardiac dysfunction,5 inflammatory conditions,6 auto-immune, and neurodegenerative diseases.7,8 However, ONOO⁻ has been proposed to act as a physiological mediator in certain contexts, for example in immune system function and ischemic preconditioning.9 Macrophages produce nitric oxide and superoxide to form ONOO⁻ to attack invading pathogens.10 Macrophage cells recognize lipopolysaccharide (LPS), a component of the bacterial cell wall, which induces high expression of inducible nitric oxide synthase (iNOS).12 The effect of LPS is amplified by release of IFN-β, providing a mechanism for robust local production of NO⁻ and ONOO⁻. Although important biological functions of ONOO⁻ are still emerging, there remains a lack of efficient and selective methods to monitor its production in real time to study its effects in living systems. Therefore, the development of precise approaches for detecting ONOO⁻ is crucial to provide a more detailed understanding of its complex biological effects.

Much of the difficulty of studying the biological roles of ONOO⁻ stems from its complex chemistry. ONOO⁻ coexists with its protonated form ONOOH (pKₐ 6.8) under physiological pH, and can directly undergo two-electron reactions with thiols,13 CO₂,14 and carbonyls.15 The H⁺ and CO₂ adducts, ONOOH and ONOOOCO₂⁻, rapidly decompose via homolytic cleavage reactions to form secondary radicals such as hydroxyl radical (HO•), carbonate radical (CO₃•⁻), and nitrogen dioxide (NO₂•), which mediate potent one-electron chemistry, including tyrosine nitration.16 ONOO⁻ can also form from reaction between O₂ and NO⁻ produced by photolysis of Angeli’s salt under alkaline conditions.17 Under physiological conditions, Angeli’s salt has been widely used as a nitroxyl (HNO) donor for biological studies. Reaction of HNO with O₂ also proceeds at neutral pH to form a potent oxidative species and represents an important decomposition pathway of HNO.18,19 While there are similarities and differences between ONOO⁻ and the autooxidation product of HNO formed at neutral pH, the details of this
Results and discussion

Design and synthesis of PNCL

As part of a program to develop chemical probes for imaging reactive sulphur, oxygen, and nitrogen species, our laboratory has previously reported that isatin reacts with ONOO\(^-\) via an oxidative decarboxylation reaction to generate an anthranilic acid.\(^\text{46}\) \(^\text{13}\)\(^\text{F}\) NMR probes, 5-fluoroisatin and 6-fluoroisatin, have been developed for selective detection of ONOO\(^-\) that utilize this newly discovered transformation. In order to expand this reaction-based detection strategy for luminogenic detection, we adopted a self-immolative 1,6-elimination strategy for chemiluminescent ONOO\(^-\) detection that relies on tethering an imidoyl moiety to a 1,2-dioxetane chemiluminescent scaffold via an ether linkage (Scheme 1). The isatin serves as the reaction handle, and 1,6-elimination can be triggered after reacting with ONOO\(^-\) to form the anthranilic acid with the amino group positioned \(\text{para}\) to the benzyl ether. Luminescent emission is then initiated upon the decomposition of the 1,2-dioxetane through a chemically initiated electron exchange luminescence (CIEEL) mechanism. Synthesis of a peroxynitrite chemiluminescent probe (PNCL) proceeded from commercially available 5-iodoisatin 1 by protection of the ketone group as the dimethyacetal 2 (Scheme 2). The protected 5-iodoisatin was subjected to palladium catalyzed reductive formylation with sodium cyanobenzyl alcohol as a sensitizer to provide the final 1,2-dioxetane PNCL.

Spectral response of the probes to ONOO\(^-\)

After obtaining PNCL, its photophysical properties and reactivity towards ONOO\(^-\) were characterized. Luminescence emission spectra were collected using an F-7000 Hitachi spectrophotometer by treating 20 \(\mu\)M PNCL with 0–200 \(\mu\)M ONOO\(^-\) (Fig. 1). An emission peak centered at 525 nm was observed in the presence of ONOO\(^-\) with \(\sim\)28-fold increase in peak luminescence intensity compared to a blank control (Fig. 1A). The reaction between PNCL and ONOO\(^-\) was analyzed by GC-MS, confirming the generation of 2-adamantamone and 2-chloro-4-acylonitrile-3-(methoxycarbonyl)phenol (Fig. S1). The luminescence emission slowly decayed over a course of 20 min (Fig. 1B), consistent with fast reaction kinetics of ONOO\(^-\) with the isatin moiety of PNCL,\(^\text{47}\) followed by a slower 1,6-elimination. Interestingly, ONOO\(^-\) can be added to a solution of PNCL over several cycles with good recovery of chemiluminescent signal (Fig. S2). This shows that the reversible chemiluminescent emission has good reproducibility and can be used monitor ONOO\(^-\) fluxes over time. The dose dependence of the peak chemiluminescent emission at 525 nm measured 10
200 μM ONOO− was compared against other analytes in HEPES buffer (20 mM, pH 7.4), by adding 5 mM reduced glutathione (GSH), 1 mM L-cysteine, or 200 μM other reactive species. Most of the species tested displayed no significant increase in luminescence intensity over the blank control within 20 min (Fig. 2). Given the destructive reactivity of HO+, we examined the interference of this species by treating PNCL with ONOO− in the presence of the HO+-generating Fenton system [Fig. S4†], where only a minimal reduction in signal was observed. A small luminescence increase was observed when treating PNCL with Angeli’s salt, commonly used as a nitroxy (HNO) donor. This was consistent with previous observations using HPLC to track the reactivity of 5-fluoroisatin with Angeli’s salt.37

In light of studies exploring the generation of ONOO− and other nitrogen oxide species from the autoxidation of NO− and HNO,16–19 we performed a series of competition experiments with HCO3− and GSH to better understand the luminescence increase resulting from reaction of PNCL with Angeli’s salt. ONOO− reacts rapidly with CO2, but slowly with GSH. HNO, on the other hand, reacts rapidly with GSH.42 We treated PNCL with ONOO− (Fig. 3A and B) or Angeli’s salt (Fig. 3C and D) in the presence of various amounts of HCO3− (1 mM, 2 mM, 3 mM, 4 mM, and 5 mM). HCO3− decreased the chemiluminescence emission intensity generated by both ONOO− and Angeli’s salt, providing evidence that Angeli’s salt can generate ONOO−. A similar experiment was performed by reacting PNCL (20 μM) with ONOO− (Fig. 4A and B) or Angeli’s salt (Fig. 4C and D) and various amounts of GSH (50 μM, 100 μM, 200 μM, and 1 mM). However, the GSH only quenched the luminescence intensity from Angeli’s salt (Fig. 4B) and no significant luminescence decrease was observed from ONOO− (Fig. 4A). Interestingly, substoichiometric quantities of GSH would not only reduce the total amount of luminescence, but displayed a lag time before producing a chemiluminescent signal (Fig. 4C). Angeli’s salt generates HNO with a half-life of 2.3 minutes at 37 °C. The observed lag time of the chemiluminescent response is

seconds after ONOO− addition showed excellent linearity, demonstrating that ONOO− can be quantified using PNCL (Fig. 1C). Calibration of the response of PNCL with lower concentrations of ONOO− revealed a detection limit of 6 nM (3σ) bolus ONOO− (Fig. S3†), comparing favorably to previous fluorescent and chemiluminescent methods (Table S1†). The reactivity of PNCL towards SIN-1 was also evaluated (Fig. 1D), showing that PNCL can monitor the slow release of ONOO− over time, as would be observed in biological systems.

The selectivity of PNCL for ONOO− was tested against a panel of biologically relevant reactive sulphur, oxygen, and nitrogen species and metal cations. The response of PNCL to

![Scheme 2](image)  
**Scheme 2** Synthesis of PNCL

![Fig. 1](image)  
**Fig. 1** Response of PNCL to ONOO−. (A) Chemiluminescent emission spectra of 20 μM PNCL and 0 (blue trace), 5, 10, 20, 40, 80, 100, 200 μM (red trace) ONOO−. (B) Time scans of the chemiluminescent emission at 525 nm of 20 μM PNCL and 0 (blue trace), 5, 10, 20, 40, 80, 100, 200 μM (red trace) ONOO−. (C) Peak emission intensity at 525 nm of 20 μM PNCL after adding 0–200 μM ONOO−. (D) Time scan of the chemiluminescent emission at 525 nm of 20 μM PNCL and 200 μM SIN-1. All experiments were performed in 20 mM HEPES (pH 7.4), containing <1% DMSO.

![Fig. 2](image)  
**Fig. 2** Selectivity of PNCL versus cations and reactive sulphur, oxygen, and nitrogen species. Chemiluminescent emission at 525 nm of 20 μM PNCL and 200 μM biologically relevant analytes in 20 mM HEPES (pH 7.4). Bars represent chemiluminescent emission at 525 nm at 4, 8, 12, 16, 20 min after addition of reactive species. Legend: (1) ONOO−, (2) Cys, (3) DEA NONOate, (4) GSH (5 mM), (5) GSNO, (6) H2O2, (7) Angeli’s salt, (8) KO2, (9) Na2S, (10) Na2S2O3, (11) Na2SO4, (12) NaNO2, (13) HOCl, (14) OCI−, (15) IBuOOH, (16) IO2−, (17) Na+, (18) Mg2+, (19) K+, (20) Ca2+, (21) Zn2+, (22) Fe2+, (23) blank. Error bars are ± S.D. All experiments were performed in 20 mM HEPES or 20 mM PBS (pH 7.4), containing <1% DMSO.
consistent with GSH scavenging the newly formed HNO more rapidly than the autooxidation reaction of HNO. Once the GSH is consumed, HNO can be oxidized to ONOO\(^-\) or another nitrogen oxide species capable of two-electron chemistry and reaction with PNCL proceeds to produce a luminescent signal.

In order to confirm this selectivity and that HCO\(_3^-\)/CO\(_2\) does not scavenge HNO, we prepared a fluorescent nitroxy (HNO) probe, herein called XF1 (Fig. 5A). HNO reacts with the diphenylphosphinobenzoyl group to form an aza-ylide that will nucleophilically attack the ester to trigger the release of the fluorophore.\(^{45,44}\) A time-dependent fluorescence turn-on was observed when treating 10 \(\mu\)M XF1 with 200 \(\mu\)M Angeli’s salt (Fig. 5A and B), and selectivity tests demonstrated that XF1 can distinguish HNO from other reactive sulfur, oxygen, and nitrogen species (Fig. 5C). Inhibition experiments were conducted by treating XF1 with various amounts of HCO\(_3^-\) or GSH together with Angeli’s salt. While no obvious inhibition of the fluorescence response was observed with the addition of HCO\(_3^-\), the fluorescence was completely quenched with 200 \(\mu\)M GSH (Fig. 5B and C). These results confirm that GSH scavenges HNO generated from Angeli’s salt but HCO\(_3^-\)/CO\(_2\) does not scavenge HNO. Taken together, the results from Fig. 3–5 are consistent with the luminescence increase from Angeli’s salt being due to the generation of ONOO\(^-\). We do note that alternative oxidative species formed from HNO have been proposed.\(^{18}\) Although we cannot completely rule this out, the results here are consistent with the formation of ONOO\(^-\) from HNO and support the high specificity of PNCL for detecting ONOO\(^-\). As a final mechanistic investigation, we examined the selectivity of ONOO\(^-\) versus NO\(_2\)\(^-\), HO\(^-\), and CO\(_2\)\(^-\) (the decomposition products of ONOO\(^-\)) by treating isatin with ONOO\(^-\) in the presence of HCO\(_3^-\)/CO\(_2\) or TEMPOL, a free radical scavenger (Fig. S6†). While HCO\(_3^-\)/CO\(_2\) inhibits the reaction, TEMPOL has no effect. This suggests that isatin reacts directly with ONOO\(^-\)
and not with free radical species like NO$_2^-$, HO', or CO$_3$$^-$ that are formed via spontaneous ONOO$^-$ decomposition.

**Evaluation of PNCL for ONOO$^-$ detection in living cells**

After demonstrating that PNCL reacts with ONOO$^-$ with high sensitivity and selectivity, we then proceeded to investigate the ability of PNCL for ONOO$^-$ detection in living cells using a BioTek Cytation 5 plate reader. Toxicity was determined by the MTT assay, which indicated low toxicity at concentrations less than 100 mM and more than 50% viability after treating with high concentrations of 1 mM PNCL for 24 hours (Fig. S7†). Cellular internalization was verified by imaging the intrinsic fluorescence emission of PNCL (Fig. S8†). In order to evaluate the ability of PNCL to detect cellular delivery of ONOO$^-$ from donor compounds, RAW 264.7 macrophages were incubated with PNCL for 30 minutes, washed, and then treated with 0, 200 mM, 400 mM, 800 mM, 1 mM, or 2 mM SIN-1, a commonly used ONOO$^-$ donor that operates by simultaneously generating NO$^-$ and O$_2$•$. A$ time course of the chemiluminescent emission intensity increased to reach a maximum at 40 minutes (Fig. 6A). Both the peak and integrated luminescence intensities increased with increasing concentrations of SIN-1 (Fig. 6A and B), and a 5-fold luminescent increase was obtained with 2 mM SIN-1 compared with a blank control. We also performed the same experiment in A549 cells (Fig. S9†). Similar results were observed and excellent linearity was achieved in living cells between 0 and 400 mM SIN-1 (Fig. S9C†). This increase in chemiluminescent emission from SIN-1 and PNCL was attenuated by the ONOO$^-$ scavenger Mn(III)TMPyP (Fig. S10†), confirming that the response is due to ONOO$^-$.

Finally, we investigated the ability of PNCL to detect ONOO$^-$ generated by macrophages stimulated to mount an immune response. Lipopolysaccharide (LPS) binds to the toll-like receptors of macrophages, activating the transcription factor NF-$\kappa$B ultimately leading to the expression of iNOS and generation of reactive oxygen species that can produce ONOO$^-$.$^{12}$ Macrophages stimulated with LPS produced increased chemiluminescent signal from PNCL (Fig. 6C), showing a 36% increase in integrated luminescence intensity with 1000 ng mL$^{-1}$ LPS. Treatment with the ONOO$^-$ scavenger Mn(III)TMPyP reduces this signal back to control levels and co-incubation with the selective iNOS inhibitor 1400 W also reduces the signal from LPS and PNCL (Fig. S11†). These control experiments validate the ability of PNCL to detect cellular production of ONOO$^-$. Bright field images of the cells after LPS stimulation show the formation of phagosomes (Fig. 6D), a phenotypic response to LPS exposure.

**Conclusions**

We have designed and synthesized a reaction-based chemiluminescent probe for ONOO$^-$, PNCL. PNCL reacts with ONOO$^-$ through an oxidative decarbonylation reaction to initiate light emission that can be observed instantly with high sensitivity and selectivity against other reactive sulphur, oxygen, and nitrogen species. The observed reactivity towards Angeli’s salt is attributed to the generation of ONOO$^-$, which is supported by inhibitor experiments with HCO$_3^-$ and GSH, providing experimental evidence of an autooxidative product of HNO. Several studies have purported that the autooxidation of HNO produces a product unique from ONOO$^-$, as evidenced by data showing no effect of HCO$_3^-$ on the reaction between Angeli’s salt and reactive oxygen species probe dihydrorhodamine 123.$^{18}$ In our studies, however, we observe a drastic HCO$_3^-$ dependent reduction in chemiluminescent signal from PNCL and Angeli’s salt, consistent with ONOO$^-$ formation from HNO under physiological conditions.$^{19}$ PNCL was used to detect ONOO$^-$ generated from donor compounds in multiple cell lines, as well as cellular ONOO$^-$ generated by macrophages stimulated with LPS. Confirmation of the chemical specificity towards ONOO$^-$ over downstream free radical products, coupled with operational compatibility in living cells establishes PNCL a useful and easy-to-use tool for studying ONOO$^-$ in chemical and biological systems.

**Conflicts of interest**

A. R. L. discloses a financial stake in BioLum Science, LLC, a company developing asthma monitoring technology.
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