Direct chemiluminescence detection of nitric oxide in aqueous solutions using the natural nitric oxide target soluble guanylyl cyclase

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A R T I C L E   I N F O

Keywords:
Guanylyl cyclase
Nitric oxide
Nitric oxide synthase
Chemiluminescence
Luciferase
Pyrophosphate
NO donors
Free radicals

A B S T R A C T

Nitric oxide (NO) is a free radical involved in many physiological processes including regulation of blood pressure, immune response, and neurotransmission. However, the measurement of extremely low, in some cases subnanomolar, physiological concentrations of nitric oxide presents an analytical challenge. The purpose of this methods article is to introduce a new highly sensitive chemiluminescence approach to direct NO detection in aqueous solutions using a natural nitric oxide target, soluble guanylyl cyclase (sGC), which catalyzes the conversion of guanosine triphosphate to guanosine 3′,5′-cyclic monophosphate and inorganic pyrophosphate. The suggested enzymatic assay uses the fact that the rate of the reaction increases by about 200 times when NO binds with sGC and, in so doing, provides a sensor for nitric oxide. Luminescence detection of the above reaction is accomplished by converting inorganic pyrophosphate into ATP with the help of ATP sulfurylase followed by light emission from the ATP-dependent luciferin–luciferase reaction. Detailed protocols for NO quantification in aqueous samples are provided. The examples of applications include measurement of NO generated by a nitric oxide donor (PAPA-NONOate), nitric oxide synthase, and NO gas dissolved in buffer. The method allows for the measurement of NO concentrations in the nanomolar range and NO generation rates as low as 100 pM/min.

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signal was amplified by using NO as a catalytic molecule rather than a direct participant in the chemical reaction and (ii) the amplified analytical signal was transformed into a chemiluminescent signal, theoretically allowing detection of a single quantum of light. The general outline of the NO detection principle is shown in Scheme 1. The amplification step was accomplished by using soluble guanylyl cyclase (sGC), a natural cellular target of nitric oxide. About a hundredfold increase in activity of sGC over the basal level occurs upon binding of NO. This increase in the rate of sGC-catalyzed conversion of guanosine triphosphate into cGMP and inorganic pyrophosphate (PPi) provides an amplification of the NO signal. The resulting pyrophosphate reacts with adenosine 5′-phosphosulfate (APS) in the presence of ATP-sulfurylase, forming ATP and sulfate; ATP then serves as a cosubstrate in the luciferase reaction, producing oxyluciferin, AMP, pyrophosphate, CO2, and a quantum of light with quantum yield about 0.4 [21]. Light output is measured by a luminometer. Essentially, by measuring chemiluminescence, we measure the kinetics of pyrophosphate formation [22], the latter being produced by NO-activated guanylyl cyclase. It is important to mention that the nanomolar concentration of ATP generated is far below the luciferase $K_m$ for ATP (160 μM [23]), which results in a linear dependence of luminescence on ATP concentration. The developed approach represents a new highly sensitive tool for detection of NO concentrations in the nanomolar range and NO generation rates in biological samples with sensitivity of 100 pM/min.

**Experimental basis**

Fig. 1 shows the linear dependence of the chemiluminescence of the luciferase system containing APS and ATP sulfurylase on the ATP or pyrophosphate concentration, in agreement with the literature data [22]. This provides an opportunity to monitor NO-dependent pyrophosphate formation by activated guanylyl cyclase. Special precautions described under Caveat have to be taken against ATP and pyrophosphate impurities in the luciferase, APS, and GTP samples to decrease the background luminescence of the luciferase/sulfurylase system.

Addition of guanylyl cyclase to the luciferase–sulfurylase reaction system results in a linear increase in luminescence for at least 10 min (Fig. 2, inset). This increase is not observed in the absence of either GTP or sGC and represents basal (not NO-stimulated) sGC activity. Addition of an NO donor (PAPA-NONOate, $t_{1/2} = 77$ min at 22 °C, pH 7.4 [24]) or bolus addition of NO dissolved in buffer to the reaction mixture dramatically increased the rate of luminescence change (Figs. 2 and 3, respectively). We observed that the kinetics of the NO-induced luminescence was strongly affected by superoxide dismutase (SOD) (Fig. 3). Addition of SOD significantly improved the sensitivity of the approach, particularly at low NO concentrations. For this reason, SOD addition to the detection system is strongly recommended for quantitative NO measurements and was used in all further applications described here. Further studies are required to identify the possible source of the superoxide production in the detection system.

![Diagram](https://via.placeholder.com/150)

**Scheme 1.** General outline of the chemiluminescence detection of nitric oxide.
A possible source is the reaction of luciferin with molecular oxygen producing dehydroluciferin and hydrogen peroxide or, possibly, superoxide as a minor product.

Materials

Note. Not all the reagents that follow are needed for each specific application of the chemiluminescence assay. Before starting, select the appropriate protocol to determine which reagents will be needed for a particular application. We recommend that GTP and APS of the highest purity grade be used and when necessary additionally purified as described below.

Enzymatic NO detection system

Chemicals

The following reagents were used: ATP (10127523001) (Roche Diagnostics, Indianapolis, IN, USA), sodium pyrophosphate (205975000), diethylenetriamine pentaacetic acid (DTPA; 114322500), diethylenetriamine pentaacetic acid (DTPA; 114322500) (Sigma-Aldrich), GTP, sulfurylase reaction mixture (Alam, 2005), aps, 1 M MgCl₂, 1 M glucose, 0.5 μl hexokinase (1.5 U/μl), 78 μl 0.1 M Tris–HCl, pH 7.5. GTP and APS concentrations in filtrate were determined spectrophotometrically using ε = 1.37 × 10⁴ M⁻¹ cm⁻¹ at 253 nm (GTP) and ε = 1.25 × 10⁴ M⁻¹ cm⁻¹ at 260 nm (APS). Treated solutions of GTP and APS were aliquotted (we used 30 μl aliquots) and kept at −80 °C.

Measurement of NOS-mediated NO generation

Chemicals

The following reagents were used: HEPES (H9897), imidazole (I5513), α-aminolevulinic acid (A7793), chloramphenicol (C0857) (Sigma–Aldrich), Terrific broth (22711–022), carbenicillin (10177–012), isopropyl-β-D-thiogalactoside (IPTG; 15529–019) (Invitrogen), NADPH (N4505), arginine (A8094) (Sigma–Aldrich), N°-monomethyl-l-arginine (NMMA; 80200), tetrahydrobiopterin (BH₄; 81880) (Cayman Chemical, Ann Arbor, MI, USA), and proteinase inhibitor cocktail tablets (11697498001) (Roche Diagnostics).

Preparation of iNOS

Overexpression of active inducible nitric oxide synthase (iNOS) in Escherichia coli was enhanced by coexpression with calmodulin (CaM). Plasmids containing iNOS and CaM/PACY were transformed into Δ65 protease–deficient E. coli BL21(DE3). The iNOS/CaM-expressing BL21 cells were cultured on LB agar plates containing carbenicillin (125 μg/ml) and chloramphenicol (35 μg/ml). One-liter cultures of Terrific broth containing 125 μg/ml carbenicillin, 35 μg/ml chloramphenicol, and 8 ml of glycerol were inoculated with 100 ml of overnight bacterial culture and shaken at 200 rpm at 37 °C. Expression of protein was induced by adding α-aminolevulinic acid to a final concentration of 500 μM and IPTG to a final concentration of 1 mM to the culture when it reached an optical density of 0.8 at 600 nm. Cells were harvested by centrifugation 20 h after induction. The cells from 4 liters of culture were resuspended in a minimum volume of lysis buffer A, containing 40 mM HEPES, 150 mM NaCl, 20 mM imidazole, 10% glycerol, 3 mM DTT, and protease inhibitor cocktail tablets at pH 7.4. Cells were lysed by two passes through an Emulsiflex C3 at 12–15 kpsi. The lysate was centrifuged at 48,000g for 60 min. The supernatant was loaded onto a 5–ml HiTrap column (GE Biosciences) and equilibrated with buffer A. The column was extensively washed with buffer B: 40 mM HEPES, 450 mM NaCl, 10% glycerol, 40 mM imidazole, 3 mM DTT, and protease inhibitor cocktail tablets at pH 7.4. Cells were lysed by two passes through an Emulsiflex C3 at 12–15 kpsi. The lysate was centrifuged at 48,000g for 60 min. The supernatant was loaded onto a 5–ml HiTrap column (GE Biosciences) and equilibrated with buffer A. The column was extensively washed with buffer B: 40 mM HEPES, 450 mM NaCl, 10% glycerol, 40 mM imidazole, 3 mM DTT, and protease inhibitor cocktail tablets at pH 7.4. The concentrated proteins were applied to a Superdex 200 Hilo column size exclusion column (GE Biosciences) and eluted with 40 mM HEPES,

Composition of the reaction mixture

Unless otherwise stated, the reaction mixture for luminescence measurements (300 μl) contained 1 mM MgCl₂, 1 mM DTE, 0.1 mM IPTG, 0.1 mg/ml β-luciferin, 0.2 μg luciferase, 0.002 U Pase, guanylyl cyclase (25–50 ng total), 0.01 U sulfurylase, 50 μl superoxide dismutase, 0.01 mM APS, and 0.1 mM GTP (both APS and GTP were treated as described above) in 0.1 M Tris–HCl, pH 7.5.

Purification of GTP and APS

To remove ATP contamination, GTP was treated as follows: a reaction mixture containing 40 μl 0.1 M GTP, 40 μl 1 M MgCl₂, 4 μl 1 M glucose, 1 μl hexokinase (1.5 U/μl), 351 μl 0.1 M Tris–HCl, pH 7.5, was incubated at room temperature for 20 min and then filtered through a Microcon Ultracel centrifuge filter YM-3 (Millipore, Bedford, MA, USA) for 20 min at 14,000 g, 4 °C for removal of hexokinase. APS was treated the same way, with a reaction mixture containing 20 μl 20 mM APS, 1 μl 1 M MgCl₂, 1 μl 1 M glucose, 0.5 μl hexokinase (1.5 U/μl), 78 μl 0.1 M Tris–HCl, pH 7.5. GTP and APS concentrations in filtrate were determined spectrophotometrically using ε = 1.37 × 10⁴ M⁻¹ cm⁻¹ at 253 nm (GTP) and ε = 1.25 × 10⁴ M⁻¹ cm⁻¹ at 260 nm (APS). Treated solutions of GTP and APS were aliquotted (we used 30 μl aliquots) and kept at −80 °C.
Measurement of NO release from NO donors

Note. PAPA-NONOate was used in the exemplified application given in this paper. A similar assay can be used to study NO release by other NO donors. A stock solution of PAPA-NONOate (82140; Cayman Chemical) was prepared in 0.01 M NaOH, and its concentration was determined using $\varepsilon = 8050 \text{ M}^{-1} \text{ cm}^{-1}$ at 250 nm. The solution is stable for 24 h.

Instrumentation

Most commercially available luminometers can be used for the luminescence measurements described. In this work all the measurements were conducted at 27 °C using an LB9505 luminometer (Berthold Analytical Instruments, Nashua, NH, USA).

Protocol for measurement of NO concentrations in aqueous solutions

Preparation of solutions

1. Five milliliters of the main buffer contains 4.84 ml of Tris–HCl (0.1 M, pH 7.5), 50 μl of DTPA (10 mM), 50 μl of BSA (10 mg/ml), 5 μl of MgCl$_2$ (1 M), 25 μl of DTE (0.2 M), 14 μl of luciferin (5 mM), 17 μl of SOD (10 mg/ml), and 1.7 μl of luciferase (2 ng/ml). Keep on ice.

2. One hundred microliters of the AGP mix (5.8 μl) and record the NO-stimulated luminescence.

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Sample preparation and luminescence recording

Put 293 μl of the main buffer into a luminometer sample tube and leave for temperature equilibration in the luminometer cell compartment for 7 min. Add the AGP mix (5.8 μl) and incubate for 2 min to allow the pyrophosphatase to hydrolyze the pyrophosphate impurity present in APS and GTP. Then, add 1 μl of sulfurylase (0.01 U). At this point luminescence recording starts. After recording the baseline luminescence for 1–2 min, add guanylyl cyclase (2 μl, 25 ng) and continue registration of luminescence. The observed gradual increase in luminescence represents the unstimulated guanylyl cyclase reaction. Add an aliquot of NO donor or NO gas solution (0.5–5 μl) and record the NO-stimulated luminescence kinetics. For the registration of NO synthase activity, the sample preparation is the same except that 10 μl of iNOS activity mix is added at the same time as the AGP mix and the reaction is started by the addition of iNOS.

Note 1. Concentrations of the GTP and APS stock solutions can vary from what is stated in the second step under Preparation of solutions; adjust the amount in the AGP mix and the amount added to the sample accordingly to have the final concentrations of 10 μM APS and 100 μM GTP in the sample.

Measurement of NO concentrations in aqueous solutions

The proposed approach provides the data in the form of the kinetics of the luminescence change. It is expected that a constant NO concentration in solution should result in a linear increase in the luminescence, as pyrophosphate generation by sGC proceeds at a constant rate. Therefore, the rate of the luminescence change calculated as a slope of the kinetics is expected to be proportional to the NO concentration and can be used for NO quantification in the sample.

Typical kinetics of the luminescence increase observed after the addition of nanomolar concentrations of anaerobic NO solution to the NO-detection system are shown in Fig. 4. The dependence of the initial rate of luminescence change on NO concentration is shown in Fig. 5. As expected, it was proportional to the NO concentration with the sensitivity limit at about 1 nM. However, the slope of the kinetic curve decreased with time for the kinetics initiated by dissolved NO gas (Figs. 3 and 4), probably owing to NO depletion in the solution. The direct uncatalyzed reaction with molecular oxygen cannot explain this decrease, as the NO first half-life at 100 nM would be about 1.5 h and even longer for lower concentrations [27,28].

Measurement of the rates of NO generation

It is expected that the rate of the initial luminescence change after initiation of NO generation will be indistinguishable from the background level owing to insufficient NO accumulation at the zero time point. On the other hand, NO generation will result in the accumulation of NO and “acceleration” of the luminescence change, which should be proportional to the NO generation rate. Therefore, luminescence acceleration calculated as the second derivative of the luminescence curve, is expected to be proportional to the NO generation rate and can be used for its quantitation.

Typical luminescence kinetics observed after addition of the NO donor, PAPA-NONOate, are shown in Fig. 6. The initial acceleration of the luminescence (second derivative of the luminescence curve at the zero time point) was proportional to the concentration of NO donor (Fig. 7), as expected. This agrees with acceleration being proportional to NO generation rate. It was also observed that the maximal rate of luminescence change is proportional to the concentration

![Fig. 4](image)

**Fig. 4.** Typical luminescence kinetics of the NO-detection system initiated by the addition of NO gas in buffer (final NO concentrations shown). The NO-detection system contains all the components of luciferase–sulfurylase reaction mixture described in the legend to Fig. 1 with the addition of pyrophosphatase (2 mU), sGC (50 ng), and SOD (50 U). Zero time points of kinetics after the addition of NO are indicated by arrows.
of NO donor (Fig. 8). This can be explained by the fact that the steady-state level of NO accumulated in solution is proportional to the rate of NO generation, i.e., to the concentration of the NO donor. The maximal rate of luminescence change observed in the presence of 3.3 nM PAPA-NONOate (corresponds to an NO release rate of about 100 pM/min) exceeded the basal rate of luminescence change by about three times.

The extraordinarily high sensitivity of the approach was further confirmed by measurement of NO generation by purified inducible nitric oxide synthase (Fig. 9). The luminescence intensity increase here depended on the presence of NADPH and arginine and was inhibited by the specific NOS inhibitor NMMA (data not shown). The rate of luminescence change was proportional to iNOS concentration, allowing for detection of NO generated by only 20 pg of purified protein. Similar shapes of the luminescence kinetics were observed upon NO release by NO donor (Fig. 6) and NO generation by iNOS (Fig. 9). The dependence of the initial acceleration of the luminescence (second derivative of the luminescence curve at the zero time point) on iNOS concentration shown in Fig. 10 exhibits the same proportionality as in the case of NO release by NO donor (cf. Figs. 10 and 7). Taking into account that the NO generation rate by PAPA-NONOate is known, the observed dependence of the initial luminescence acceleration on the concentration of NO donor (Fig. 7) can be used as a calibration curve for the calculation of the rate of NO production by iNOS (Fig. 10, left axis). The dependence of NO generation rate versus iNOS amount shown in Fig. 10 yields the activity of iNOS equal to 1100 nmol NO\(\cdot\)min\(^{-1}\)\cdot mg\(^{-1}\). This value is in good agreement with the activity obtained by oxyhemoglobin assay, 800 nmol NO\(\cdot\)min\(^{-1}\)\cdot mg\(^{-1}\).

**Caveat: background luminescence**

The reaction mixture without sGC demonstrates background luminescence originating from ATP and pyrophosphate impurities in luciferase, APS, and GTP. Of these, the predominant problem is ATP and pyrophosphate contamination in GTP, as it is present in the highest concentration (0.1 mM) in the reaction mixture. ATP (and possibly APS) is also a substrate for luciferase, as has been shown previously [29]. To find out how much this side reaction of luciferase can add to the background luminescence, we added hexokinase and glucose to the luciferase reaction system containing either ATP or GTP (Fig. 11). ATP-depleting hexokinase activity reduces luminescence to the background level in the reaction mixture containing ATP. However, luminescence drops substantially, but not to the background, in the presence of GTP. As GTP is not a substrate for hexokinase, the drop in luminescence for the GTP-containing sample after addition of hexokinase shows that contaminating ATP contributes mainly to the GTP-originating fluorescence. Still, some luminescence is produced by GTP serving as an alternative substrate for luciferase (residual hexokinase-resistant luminescence in GTP-containing sample). Taking into account the higher GTP concentration, we conclude that GTP is about 14,000 times less effective at activating luciferase luminescence than ATP. In further experiments GTP and APS were treated with hexokinase as described under Materials.

Commercial GTP preparations also contain substantial amounts of pyrophosphate. This was found by observing a sharp increase in the luminescence of the luciferase reaction system containing GTP upon addition of APS and ATP sulfurylase. To remove initially present pyrophosphate, a small amount of pyrophosphatase was added to the reaction mixture before the addition of sulfurylase, as described under
Sample preparation. The added amount was adjusted to be sufficient to hydrolyze practically all the contaminating pyrophosphate in about 2 min before sulfurylase addition. Note that in the presence of sulfurylase most of pyrophosphate produced in the guanylyl cyclase reaction is converted to ATP rather than to phosphate owing to the predominant activity of sulfurylase over pyrophosphatase. Separate experiments proved that the amount of pyrophosphatase included in the reaction mixture does not significantly affect the luminescence kinetics (data not shown).

Concluding remarks

Here we presented a new method for the measurement of nitric oxide concentrations and rates of NO generation by using a natural target of nitric oxide, soluble guanylyl cyclase. This method allows for monitoring of the guanylyl cyclase reaction, both basal and NO-stimulated, using a highly sensitive chemiluminescence detection technique. A high degree of stimulation of the sGC reaction by NO provides the way for detection of nanomolar concentrations of nitric oxide and rates of NO generation as low as 0.1 nM/min. The stimulation ratio in our experiments was in the range of 140 to 160.

The proposed chemiluminescence approach can be useful for mechanistic studies of the guanylyl cyclase reaction. To our best knowledge, this is the first method describing continuous registration of guanylyl cyclase kinetics. As can be seen from Fig. 5, the rate of the guanylyl cyclase reaction detected by the chemiluminescence method linearly depends on an NO concentration in the nanomolar range. This means that the apparent dissociation constant for NO cannot be lower than 10 nM, which is in agreement with some literature data [35–37] and contradicts others [34]. Luminescence kinetics under conditions of maximal stimulation by NO (3–5 μM PAPA-NONOate) allowed for the calculation of the specific activity of guanylyl cyclase in the reaction mixture, which was 1.8×10^3 nmol PPi/min (mg GC−1) at 27 °C. The manufacturer reports about 10^4 nmol cGMP min−1·mg−1 at 37 °C. Taking into account a 10 °C temperature difference and possible suboptimal conditions for enzyme activity (buffer composition, GTP concentration, etc.) the agreement seems to be reasonable. It should be noted that the assay described here can be also used to study NO-independent regulation mechanisms of sGC (e.g., by CO and YC-1, a synthetic benzylindazole derivative [38]) or particulate GC [39].

To adapt the technique for in vivo and in situ applications, a few problems must be resolved. First, as the method is based on the detection of ATP and PPi, molecules that are ubiquitous in the biological milieu, the whole detection system should be encapsulated, e.g., in liposomes [40]. As the liposomal membrane is permeable to nitric oxide, but not ATP and PPi, detection is possible in this configuration. Second, in vivo applications can be hampered by strong absorption of 560-nm light, emitted by firefly luciferase, in biological tissues. Recently constructed luciferase mutants [23,41] emit at 615 nm, fitting perfectly into the spectral window (ca. 600–710 nm) in which such emissions can be measured in living tissues.

Acknowledgments

This work was supported by NIH Grants KO1 EB03519, CA132068, HL38324, and HL63744 and a Faculty Research Grant from Valdosta State University. The authors express their special thanks to Dr. Alexandre Samouilov for fruitful discussions and help with some experiments.

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