Free Radical Biology & Medicine 47 (2009) 1339–1345

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Methods in Free Radical Biology & Medicine

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

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ARTICLE INFO

Article history:
Received 5 June 2009
Revised 30 August 2009
Accepted 6 September 2009
Available online 12 September 2009

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

ABSTRACT

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.

Nitric oxide (NO) plays an important role as a signaling molecule in smooth muscle tonus regulation [1], neurotransmission [2], and immune response [3–5]. That is why measurement of nitric oxide production both in vitro and in vivo is of great interest and is the subject of numerous publications [6–9]. However, the measurement of extremely low, in some cases subnanomolar, physiological concentrations of nitric oxide [10–12] presents an analytical challenge. The problem is aggravated by the reactivity of NO toward oxygen and many other substances in the biological milieu.

The primary methods currently in use for NO detection in biological samples are the chemiluminescent reaction with ozone [10–12], electrochemical [9,13], fluorescence [14–16], and electron paramagnetic resonance (EPR) approaches [17,18]. All these methods have their own drawbacks, which have been thoroughly discussed in the literature. Ozone chemiluminescence is more suited to gaseous samples, whereas electrochemical detection suffers from instability and low sensitivity of electrodes. Fluorescence detection, though highly sensitive, requires a high concentration of the probe, yields unstable fluorescent products, and can be misinterpreted, as the probe reacts with ascorbic or dehydroascorbic acid yielding a product with a fluorescent spectrum similar to one used for NO detection [19,20]. EPR approaches have a certain advantage, allowing NO measurements in living tissues, but often lack sensitivity [17,18].

One preferred way to overcome the transient nature of nitric oxide in biological samples is to measure the stable products of its metabolism, nitrate and nitrite. However, this gives only an averaged picture of NO generation, without information on instantaneous NO concentration or the rate of NO generation, which can be physiologically important.

The objective of this methods paper is to introduce a new highly sensitive chemiluminescence approach to NO detection in aqueous solutions and to provide detailed experimental protocol for its application.

Principles

The concept

To develop the method for detection of ultralow concentrations of nitric oxide we utilized the following principles: (i) an analytical

Abbreviations: APS, adenosine 5′-phosphosulfate; BH_4, tetrahydrobiopterin; BSA, bovine serum albumin; cGMP, guanosine 3′,5′-cyclic monophosphate; DTE, 1,4-dithioerythritol; DTPA, diethylenetriamine pentaacetic acid; DTT, dithiothreitol; iNOS, inducible nitric oxide synthase; IPTG, isopropyl-β-D-thiogalactoside; NMMA, N^G-monomethyl-L-arginine; PAPA-NONOate, propylamine propylamine NONOate; PP_i, inorganic pyrophosphate; PPase, inorganic pyrophosphatase; sGC, soluble guanylyl cyclase; SOD, superoxide dismutase; AGP mix, the mixture of APS, GTP, and pyrophosphatase.

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doi:10.1016/j.freeradbiomed.2009.09.007

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 (PP_i) 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 oxoluciferin, AMP, pyrophosphate, CO_2, 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.

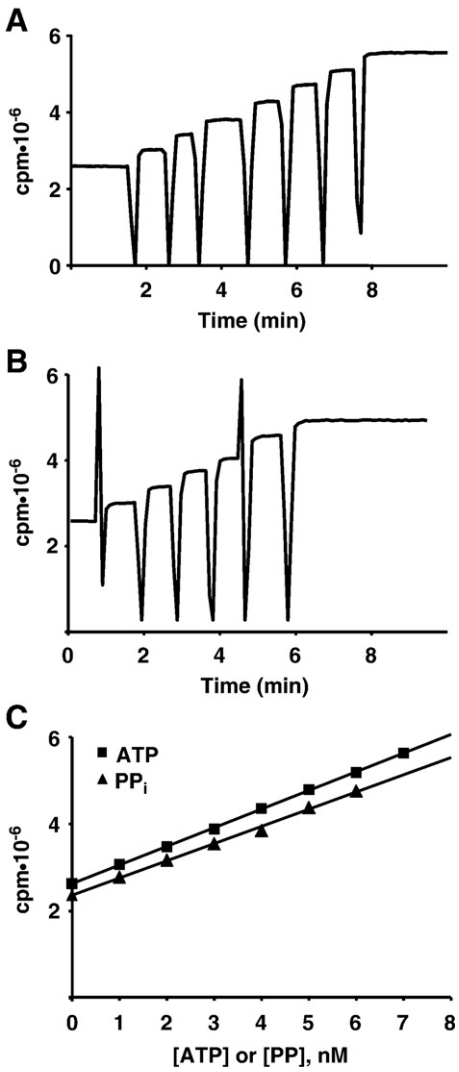
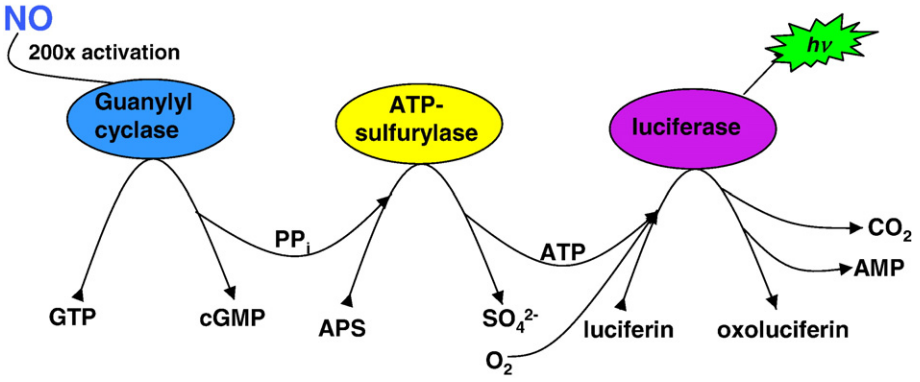


Fig. 1. Luminescence of the luciferase–sulfurylase reaction system upon addition of (A) ATP or (B) pyrophosphate; each addition is 1 nM. Spikes are from removing and replacing the sample and should be disregarded. (C) Dependence of luminescence on the concentration of ATP or pyrophosphate (nM range). The samples contained 1 mM $MgCl_2$, 1 mM DTE, 0.1 mM DTPA, 0.1 mg/ml BSA, 0.014 mM D-luciferin, 0.01 mM APS, 0.1 mM GTP (APS and GTP were treated as described under Materials), 0.2 μg luciferase, 0.01 U sulfurylase in 0.1 M Tris–HCl, pH 7.5.



Scheme 1. General outline of the chemiluminescence detection of nitric oxide.

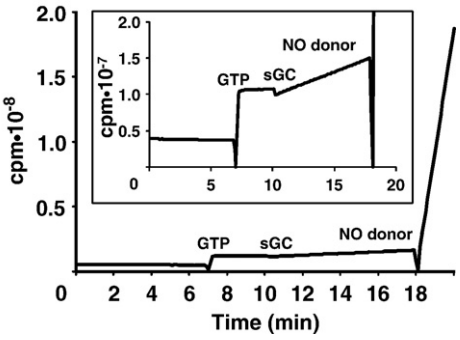


Fig. 2. Luminescence of the luciferase–sulfurylase reaction system (the same as in Fig. 1, except using GTP) upon addition of 0.1 mM GTP treated as described under Materials, guanylyl cyclase (50 ng), and NO donor (PAPA-NONOate, 67 μM). Inset: 10× expansion of vertical scale.

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) (Acros Organics), D-luciferin (L9504), GTP (G8877), APS (A5508), Trizma base (T1503), and dithiothreitol (DTT; D0632) (Sigma–Aldrich).

Enzymes

The following enzymes were used: guanylyl cyclase (ALX-202-039, soluble, bovine lung, 10 μmol cGMP/min per milligram of protein; Alexis Biochemicals, San Diego, CA, USA), ATP sulfurylase (M0394S, recombinant, from *Saccharomyces cerevisiae,* 300 units/ml; New England Biolabs, Ipswich, MA, USA), inorganic pyrophosphatase (10108987001, 200 U/mg), hexokinase (11426362001, 450 U/mg) (Roche Diagnostics), firefly luciferase (L9009, lyophilized powder, ~10^7 light units/mg protein), superoxide dismutase (S5395, from bovine erythrocytes, 5000 U/mg) (Sigma–Aldrich).

Composition of the reaction mixture

Unless otherwise stated, the reaction mixture for luminescence measurements (300 μl) contained 1 mM $MgCl_2$, 1 mM DTE, 0.1 mM DTPA, 0.1 mg/ml BSA, 0.014 mM D-luciferin, 0.2 μg luciferase, 0.002 U PPase, guanylyl cyclase (25–50 ng total), 0.01 U sulfurylase, 50 U superoxide dismutase, 0.01 mM APS, and 0.1 mM GTP (both APS and GTP were treated as described below) 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, 4.0 μl 1 M $MgCl_2$, 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 $MgCl_2$, 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 $\varepsilon = 1.37 \times 10^4\ M^{-1}\ cm^{-1}$ at 253 nm (GTP) and $\varepsilon = 1.25 \times 10^4\ M^{-1}\ cm^{-1}$ 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), δ-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^G-monomethyl-L-arginine (NMMA; 80200), tetrahydrobiopterin (BH_4; 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/pACYC 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 δ-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 L 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 48,000*g* for 60 min. The supernatant was loaded onto a 5-ml HisTrap 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, pH 7.4. Bound protein was eluted with buffer C: 40 mM HEPES, 450 mM NaCl, 10% glycerol, 250 mM imidazole, 3 mM DTT, pH 7.4. Fractions containing iNOS were pooled and concentrated using an Amicon Ultra 100,000 MW cutoff concentrator (Millipore). The concentrated proteins were applied to a Superdex 200 Hiload size exclusion column (GE Biosciences) and eluted with 40 mM HEPES,

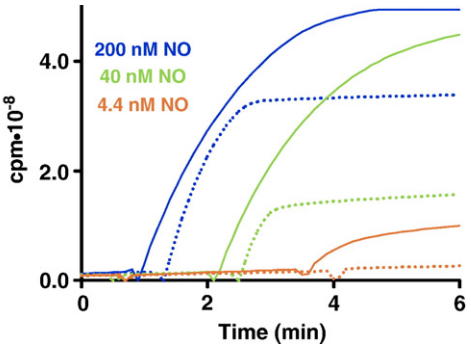


Fig. 3. Influence of SOD on the luminescence kinetics of the NO-detection system. The samples contain all the components of the luciferase–sulfurylase reaction mixture described in the legend to Fig. 1 with the addition of pyrophosphatase (2 mU) and sGC (50 ng). The kinetics were measured in the presence of SOD (50 U, solid lines) or its absence (dashed lines) and initiated by the addition of aliquots of an anaerobically prepared solution of NO gas in buffer (final NO concentrations shown).

150 mM NaCl, 10% glycerol, pH 7.4. The iNOS fractions were concentrated, divided into aliquots, quickly frozen in liquid nitrogen, and stored at −80 °C. The iNOS concentration was determined using the Bradford assay (Bio-Rad) with BSA as the standard. The purity of the iNOS was above 90% as determined by SDS–PAGE with Coomassie blue staining. The activity of iNOS was determined using the oxyhemoglobin capture assay [25], the typical activity being above 800 nmol mg^{-1} min^{-1}.

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 $\varepsilon = 8050$ M^{-1} 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 mg/ml). Keep on ice.
2. One hundred microliters of APS, GTP, and pyrophosphatase mixture (AGP mix) contains 52 μl of GTP (10 mM, treated as described), 13 μl of APS (4 mM, treated as described), and 35 μl of pyrophosphatase (1 U/ml). Keep this solution on ice for no more than 3 h owing to the instability of APS.
3. One hundred microliters of NO synthase activity mixture (iNOS activity mix) contains 63 μl of arginine (10 mM), 6.3 μl of NADPH (100 mM), and 31 μl of BH_4 (10 mM). Keep on ice for no more than 3 h owing to the oxidation of BH_4.

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.

Note 2. The addition of calmodulin to the reaction mixture does not change the iNOS activity and the addition of calcium inhibits guanylyl cyclase [26].

Note 3. Inducible NOS was used in this application. A similar assay can be used to study NO generation by other NOS isoforms.

Calculations and results

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

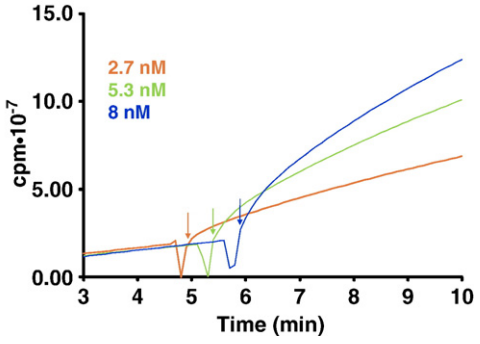


Fig. 4. Typical luminescence kinetics of the NO-detection system initiated by the addition of NO gas in buffer (final NO concentrations shown). The NO-detection system contains all the components of luciferase–sulfurylase reaction mixture described in the legend to Fig. 1 with the addition of pyrophosphatase (2 mU), sGC (50 ng), and SOD (50 U). Zero time points of kinetics after the addition of NO are indicated by arrows.

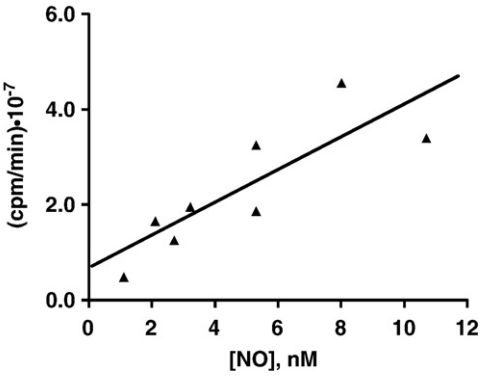


Fig. 5. The dependence of the initial rate of the luminescence increase in the NO-detection system on the concentration of NO. The rates were calculated by fitting the first minute of kinetics with the quadratic function and calculating the derivative at the zero time point (see Fig. 4 for the typical kinetics).

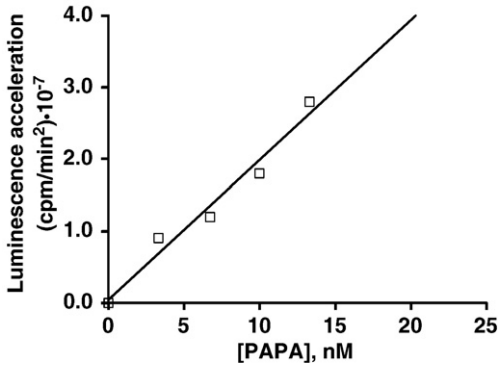


Fig. 7. Dependence of the initial acceleration of the luminescence of the NO-detection system on the concentration of PAPA-NONOate. The acceleration was calculated as the second derivative of the kinetic curve at the zero time point (see Fig. 6 for the typical kinetics).

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·min^{-1}·mg^{-1}. This value is in good agreement with the activity obtained by oxyhemoglobin assay, 800 nmol NO·min^{-1}·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. GTP (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

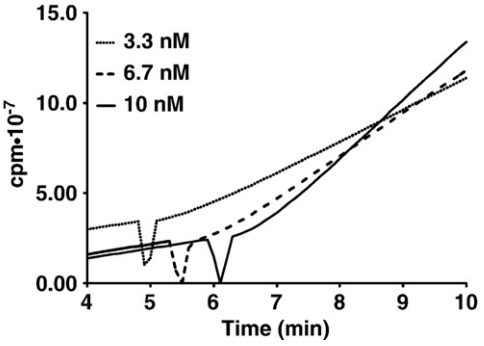


Fig. 6. Luminescence kinetics of the NO-detection system in the presence of various concentrations of PAPA-NONOate. The NO-detection system composition was the same as described in the legend to Fig. 4. Reaction was started by the addition of PAPA-NONOate.

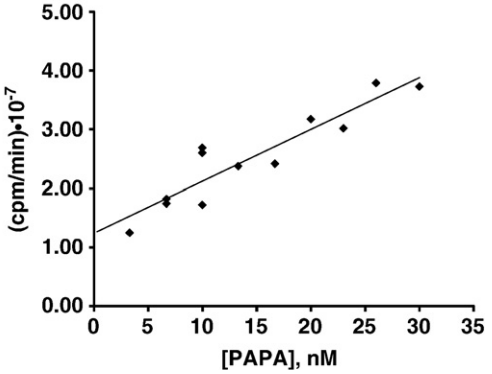


Fig. 8. Dependence of the maximal rate of luminescence increase in the NO-detection system on PAPA-NONOate concentration calculated from the kinetic curves (see Fig. 6 for the typical kinetics).

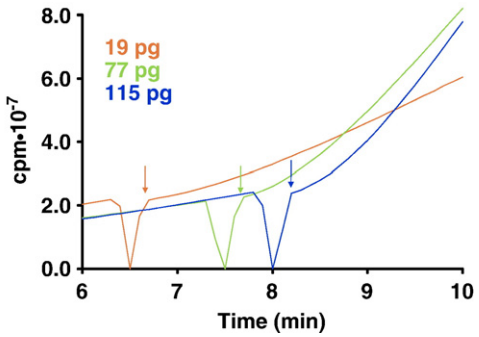


Fig. 9. Luminescence kinetics of the NO-detection system in the presence of various amounts of iNOS. The NO-detection system composition was the same as described in the legend to Fig. 4 with the addition of the components required for iNOS activity (arginine, 0.2 mM; NADPH, 0.2 mM; BH_4, 0.1 mM). Zero time points of kinetics after the addition of iNOS are indicated by arrows.

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 times, which is comparable to values (130–670) reported in the literature [30–33]. It should be noted that the basal guanylyl cyclase activity observed in our experiments was significantly lower than that reported in a recent publication [34], namely 10–40 nmol PP_i/(mg GC · min), depending on the batch of the enzyme, and did not vary substantially from day to day. This could reflect, according to the authors of mentioned publication, the quality of air (NO level) in Columbus, Ohio vs London, UK.

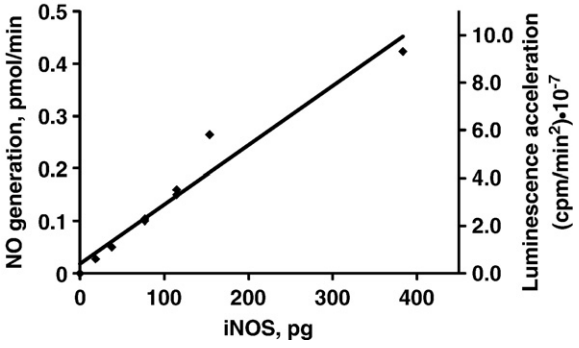


Fig. 10. Dependence of the initial acceleration of the luminescence of the NO-detection system (right axis) and rate of NO generation by iNOS (left axis) on the amount of iNOS present. Initial acceleration was calculated as a second derivative of the kinetic curve at the zero time point (see Fig. 9). The rate of NO production by iNOS was calculated based on the known rate of NO production by PAPA-NONOate (see text for details).

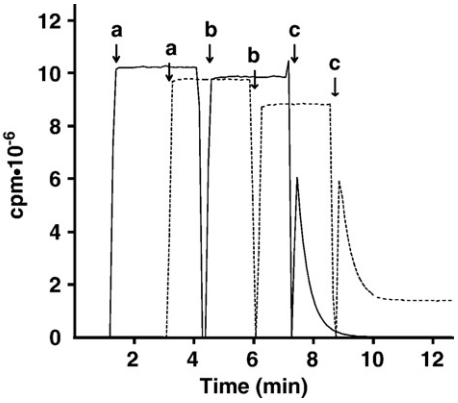


Fig. 11. Luminescence of the luciferase reaction system in the presence of ATP (solid line) or GTP (dashed line). Indicated by arrows are time points of additions of (a) ATP (40 nM) or GTP (80 μM); (b) glucose (3 mM); and (c) hexokinase (0.15 U). Reaction mixtures before additions contained 1 mM $MgCl_2$, 1 mM DTE, 0.1 mM DTPA, 0.1 mg/ml BSA, 0.014 mM D-luciferin, 0.2 μg luciferase in 0.1 M Tris–HCl, pH 7.5.

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 (3–8 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 PP_i $min^{-1} \cdot mg^{-1}$ at 27 °C. The manufacturer reports about 10^4 nmol cGMP $min^{-1} \cdot mg^{-1}$ 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 PP_i, 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 PP_i, 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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